

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: CHUNTHARAPAI ET AL.
Docket: 11669.19US03
Title: DR4 ANTIBODIES AND USES THEREOF

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number: EL440285324US

Date of Deposit: May 28, 1999

I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By: Tim JustName: Tim Just

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

We are transmitting herewith the attached:

- ☒ Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
- ☒ Continuation-In-Part Utility Patent Application: Spec. 36 pgs; 32 claims; Abstract 1 pg.
The fee has been calculated as shown below in the 'Claims as Filed' table.
- ☒ 19 sheets of informal drawings
- ☒ An unsigned Combined Declaration and Power of Attorney
- ☒ A check in the amount of \$1600.00 to cover the Filing Fee
- ☒ Return postcard

CLAIMS AS FILED

Number of Claims Filed		In Excess of:		Number Extra		Rate		Fee
Basic Filing Fee								\$760.00
Total Claims								
32	-	20	=	12	x	18.00	=	\$216.00
Independent Claims								
11	-	3	=	8	x	78.00	=	\$624.00
MULTIPLE DEPENDENT CLAIM FEE								\$0.00
TOTAL FILING FEE								\$1600.00

Please charge any additional fees or credit overpayment to Deposit Account No. 13-2725. A duplicate of this sheet is enclosed.

MERCHANT & GOULD P.C.
3100 Norwest Center, Minneapolis, MN 55402
(612) 332-5300

By: Diane L. Marschang
Name: Diane L. Marschang
Reg. No.: 35,600
Initials: DLM:PSTbkh

DR4 ANTIBODIES AND USES THEREOF

RELATED APPLICATIONS

10 This is a continuation-in-part application of pending application serial
no. 09/237,299 filed January 25, 1999, which claims priority under Section
119(e) to provisional application number 60/072,481 filed January 26, 1998,
now abandoned, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

15 The present invention relates generally to DR4 antibodies, including
antibodies which may be agonistic, antagonistic or blocking antibodies.

BACKGROUND OF THE INVENTION

20 Control of cell numbers in mammals is believed to be determined, in part,
by a balance between cell proliferation and cell death. One form of cell
death, sometimes referred to as necrotic cell death, is typically characterized
as a pathologic form of cell death resulting from some trauma or cellular
injury. In contrast, there is another, "physiologic" form of cell death which
usually proceeds in an orderly or controlled manner. This orderly or
25 controlled form of cell death is often referred to as "apoptosis" [see, e.g.,
Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science,
267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many
physiological processes, including embryonic development and clonal selection
in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels
30 of apoptotic cell death have been associated with a variety of pathological
conditions, including cancer, lupus, and herpes virus infection [Thompson,
Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may
be associated with a variety of other pathological conditions, including AIDS,
Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis,
35 multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic
anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced
liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more
characteristic morphological and biochemical changes in cells, such as

condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

Various molecules, such as tumor necrosis factor- α ("TNF- α "), tumor necrosis factor- β ("TNF- β " or "lymphotoxin- β "), lymphotoxin- β ("LT- β "), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); WO 97/25428 published July 17, 1997; WO 97/01633 published January 16, 1997; Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); Wiley et al., Immunity, 3:673-682 (1995); Browning et al., Cell, 72:847-856 (1993); Armitage et al. Nature, 357:80-82 (1992)]. Among these molecules, TNF- α , TNF- β , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- α and TNF- β have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- α is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their function is no longer needed

[Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- α [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

5 Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 10 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive 15 polymorphisms have been associated with both TNF receptor genes [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

20 The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH₂-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the 25 approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino 30 acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

35 A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell 40 antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen

OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., Cell, 66:233-243 (1991)]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- α or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- α , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, most receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- α , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. Such newly identified members of the TNFR family include CAR1, HVEM and osteoprotegerin (OPG) [Brojatsch et al., Cell, 87:845-855 (1996); Montgomery et

al., Cell, 87:427-436 (1996); Marsters et al., J. Biol. Chem., 272:14029-14032 (1997); Simonet et al., Cell, 89:309-319 (1997)]. Unlike other known TNFR-like molecules, Simonet et al., supra, report that OPG contains no hydrophobic transmembrane-spanning sequence.

5 In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has
10 also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 cDNA encodes an
15 open reading frame of 468 amino acids with features characteristic of a cell surface receptor. Pan et al. describe a putative signal peptide present at the beginning of the molecule (amino acids -23 to -1), with the mature protein predicted to start at amino acid 24 (Ala). Residues 108 to 206 contain two cysteine-rich pseudorepeats that resemble corresponding regions in TNFR-1 (four repeats), DR3 (four repeats), Fas (three repeats) and CAR1 (two repeats).
20 Following the transmembrane domain is an intracellular region containing a 70 amino acid stretch with similarity to the death domains of TNFR1, DR3, Fas, and CAR1. The DR4 transcript was detected in spleen, peripheral blood leukocytes, small intestine, and thymus. In addition, DR4 expression was also found in K562 erythroleukemia cells, MCF7 breast carcinoma cells and activated T cells.
25 Pan et al. further disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

In Sheridan et al., Science, 277:818-821 (1997) and Pan et al., Science, 277:815-818 (1997), another molecule believed to be a receptor for the Apo-2
30 ligand (TRAIL) is described. That molecule is referred to as Apo-2 (it has also been alternatively referred to as DR5). Like DR4, Apo-2 is reported to contain a cytoplasmic death domain and be capable of signaling apoptosis.

In Sheridan et al., supra, a receptor called DcR1 (or alternatively, Apo-2DcR) is disclosed as being a potential decoy receptor for Apo-2 ligand
35 (TRAIL). Sheridan et al. report that DcR1 can inhibit Apo-2 ligand function *in vitro*. See also, Pan et al., supra, for disclosure on the decoy receptor referred to as TRID.

In Marsters et al., Curr. Biol., 7:1003-1006 (1997), a receptor referred to as DcR2 is disclosed. Marsters et al. report that DcR2 contains a
40 cytoplasmic region with a truncated death domain and can function as an inhibitory Apo-2L receptor *in vitro*.

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997); Wang et al., Cell, 90:1-20 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell, 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- κ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signaling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the *Ced-3*-related protease, MACH α /FLICE (caspase 8), into the death signaling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH α /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 β converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death program [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-

KB [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-KB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-KB is complexed with members of the IKB inhibitor family; upon inactivation of the I KB in response to certain stimuli, released NF-KB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

SUMMARY OF THE INVENTION

The invention provides DR4 antibodies which are capable of specifically binding to DR4. Preferred DR4 antibodies are capable of modulating biological activities associated with DR4 and/or Apo-2 ligand, in particular, apoptosis, and thus are useful in the treatment of various diseases and pathological conditions, including cancer. In one embodiment of the invention, the DR4 antibody is a monoclonal antibody.

The invention also provides hybridoma cell lines which produce DR4 monoclonal antibodies.

The invention also provides compositions comprising one or more DR4 antibodies and a carrier, such as a pharmaceutically-acceptable carrier. In one embodiment, such composition may be included in an article of manufacture or kit.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence (SEQ ID NO:2) of a cDNA for human DR4 and its derived amino acid sequence (SEQ ID NO:1). The respective nucleotide and amino acid sequences for human DR4 are also reported in Pan et al., Science, 276:111 (1997).

Figures 2 shows the FACS analysis of two anti-DR4 antibodies, 4E7.24.3 ("4E7") and 4H6.17.8 ("4H6") (illustrated by the bold lines) as compared to IgG controls (dotted lines). Both antibodies recognized the DR4 receptor expressed in human 9D cells.

Figure 3 is a graph showing percent (%) apoptosis induced in 9D cells by DR4 antibodies, 4E7.24.3 and 4H6.17.8.

Figure 4 is a bar diagram showing percent (%) apoptosis, as compared to Apo-2L, in 9D cells by DR4 antibodies, 4E7.24.3 and 4H6.17.8, in the presence or absence of goat anti-mouse IgG Fc.

Figure 5 is a bar diagram illustrating the ability of DR4 antibody 4H6.17.8 to block the apoptosis induced by Apo-2L in 9D cells.

Figure 6 is a graph showing results of an ELISA testing binding of DR4 antibodies, 4E7.24.3 and 4H6.17.8, to DR4 and to other known Apo-2L receptors referred to as Apo-2, DcR1, and DcR2.

Figure 7 shows the binding affinities of DR4 antibodies, 4E7, 4H6, and 5G11.17.1 ("5G11"), to DR4-IgG, as determined in a KinExA™ assay. Binding affinities, e.g., of DR4 and DR5 immunoadhesins to Apo-2L are shown for comparison.

Figure 8A shows graphs illustrating percent (%) apoptosis (as determined by FACS analysis) induced in 9D cells by various concentrations of DR4 antibodies 1H5.25.9 ("1H5"), 4G7.18.8 ("4G7"), and 5G11, in the absence or presence of goat anti-mouse IgG Fc or rabbit complement.

Figure 8B shows graphs illustrating apoptotic activity (as determined by FACS analysis) of DR4 antibodies 4G7 and 5G11 on 9D cells in the presence of goat anti-mouse IgG Fc or rabbit complement.

Figure 9 shows apoptotic activity of DR4 antibodies, 4H6, 4E7, 4G7, 4G10.20.6 ("4G10"), 3G1.17.2 ("3G1"), 5G11, 1H8.17.5 ("1H8"), and 1H5.24.9 ("1H5") on SKMES colon tumor cells in the presence of goat anti-mouse IgG Fc.

Figure 10A shows apoptotic activity of DR4 antibodies 4G7 and 5G11 on SKMES colon tumor cells in the presence or absence of goat anti-mouse IgG Fc.

Figure 10B shows apoptotic activity of DR4 antibodies, 4G7 and 5G11, on SKMES colon tumor cells in the presence or absence of rabbit complement.

Figure 11A shows apoptotic activity of DR4 antibodies, 4G7 and 5G11, on HCT116 colon tumor cells in the presence or absence of goat anti-mouse IgG Fc.

Figure 11B shows apoptotic activity of DR4 antibodies, 4G7 and 5G11, on HCT116 colon tumor cells in the presence or absence of rabbit complement.

Figure 12 shows the results of a PARP assay.

Figure 13 shows the effects of DR4 antibodies, 4G7 and 5G11, on the growth of HCT116 colon tumors in athymic nude mice, as measured by tumor volume.

Figure 14 shows the effects of DR4 antibodies, 4G7 and 5G11, on the growth of HCT116 colon tumors in athymic nude mice, as measured by tumor weight.

Figures 15 and 16 show the effects of DR4 antibodies, 4G7 and 4H6, on the growth of Colo205 colon tumors in athymic nude mice, as measured by tumor volume.

Figure 17 provides a table identifying DR4 antibodies 1H5.24.9; 1H8.17.5; 3G1.17.2; 4E7.24.3; 4G7.18.8; 4H6.17.8; 4G10.20.6; and 5G11.17.1, as well as various properties and activities identified with each respective antibody.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

As used herein, the term "Apo-2 ligand" or "Apo-2L" (also known as TRAIL) refers to a specific member of the tumor necrosis factor (TNF) ligand family that induces apoptosis in a variety of cell lineages [see WO 97/25428 published July 17, 1997; Pitti et al., *J. Biol. Chem.*, 271:12687 (1996); Marsters et al., *Curr. Biol.*, 6:79 (1997); Wiley, S. et al., *Immunity*, 3:637 (1995)].

A receptor for Apo-2L has been identified and referred to as DR4, a member of the TNF-receptor family that contains a cytoplasmic "death domain" capable of engaging the cell suicide apparatus [see Pan et al., *Science*, 276:111 (1997)]. The term "Death Receptor 4" or "DR4" when used herein encompasses native sequence DR4 and DR4 variants (which are further defined herein). These terms encompass DR4 expressed in a variety of mammals, including humans. DR4 may be endogenously expressed as occurs naturally in a variety of human tissue lineages, or may be expressed by recombinant or synthetic methods. A "native sequence DR4" comprises a polypeptide having the same amino acid sequence as a DR4 derived from nature. Thus, a native sequence DR4 can have the amino acid sequence of naturally-occurring DR4 from any mammal. Such native sequence DR4 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence DR4" specifically encompasses naturally-occurring truncated or secreted forms of the DR4 (e.g., a soluble form containing, for instance, an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the DR4. In one embodiment of the invention, the native sequence DR4 is a mature or full-length native sequence DR4 comprising amino acids 1 to 468 of Fig. 1 (SEQ ID NO:1).

The terms "extracellular domain" or "ECD" herein refer to a form of DR4 which is essentially free of the transmembrane and cytoplasmic domains of DR4. Ordinarily, DR4 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally, DR4 ECD will comprise amino acid residues 1 to 218 or residues 24 to 218 of Fig. 1 (SEQ ID NO:1).

"DR4 variant" means a biologically active DR4 having at least about 80% or 85% amino acid sequence identity with the DR4 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human DR4. Such DR4 variants include, for instance, DR4 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an DR4 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95%

amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1).

"Percent (%) amino acid sequence identity" with respect to the DR4 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the DR4 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the DR4 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

The terms "agonist" and "agonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, substantially inducing, promoting or enhancing DR4 biological activity or activation.

The terms "antagonist" and "antagonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, substantially counteracting, reducing or inhibiting DR4 biological activity or DR4 activation.

The term "antibody" is used in the broadest sense and specifically covers single anti-DR4 monoclonal antibodies (including agonist, antagonist, and neutralizing or blocking antibodies) and anti-DR4 antibody compositions with polypepitopic specificity. "Antibody" as used herein includes intact immunoglobulin or antibody molecules, polyclonal antibodies, multispecific antibodies (i.e., bispecific antibodies formed from at least two intact

antibodies) and immunoglobulin fragments (such as Fab, F(ab')₂, or Fv), so long as they exhibit any of the desired agonistic properties described herein.

Antibodies are typically proteins or polypeptides which exhibit binding specificity to a specific antigen. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains [Chothia et al., J. Mol. Biol., 186:651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA, 82:4592-4596 (1985)]. The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (6) and lambda (8), based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

"Antibody fragments" comprise a portion of an intact antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments, diabodies, single chain antibody molecules, and multispecific antibodies formed from antibody fragments.

The term "variable" is used herein to describe certain portions of the variable domains which differ in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely

adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies [see Kabat, E.A. et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, MD (1987)]. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include chimeric, hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-DR4 antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as

Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the
5 recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found
10 neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-
15 human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes
20 herein mean having the ability to modulate DR4 activity or DR4 activation, including, by way of example, apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense
25 and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability
30 assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "cancer," "cancerous," and "malignant" refer to or describe the physiological condition in mammals that is typically characterized by
unregulated cell growth. Examples of cancer include but are not limited to,
35 carcinoma, including adenocarcinoma, lymphoma, blastoma, melanoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, Hodgkin's and non-Hodgkin's lymphoma, pancreatic cancer, glioblastoma, cervical cancer, glioma, ovarian cancer, liver cancer such as hepatic carcinoma
40 and hepatoma, bladder cancer, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer such as renal

cell carcinoma and Wilms' tumors, basal cell carcinoma, melanoma, prostate cancer, vulval cancer, thyroid cancer, testicular cancer, esophageal cancer, and various types of head and neck cancer.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

II. Compositions and Methods of the Invention

A. DR4 Antibodies

In one embodiment of the invention, DR4 antibodies are provided. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies. These antibodies may be agonists, antagonists or blocking antibodies.

1. Polyclonal Antibodies

The antibodies of the invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the DR4 polypeptide (or a DR4 ECD) or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for DR4 antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The antibodies of the invention may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods,

such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the DR4 polypeptide (or a DR4 ECD) or a fusion protein thereof, such as a DR4 ECD-IgG fusion protein. The immunizing agent may alternatively comprise a fragment or portion of DR4 having one or more amino acids that participate in the binding of Apo-2L to DR4. In a preferred embodiment, the immunizing agent comprises an extracellular domain sequence of DR4 fused to an IgG sequence, such as described in Example 1.

Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Manassas, Virginia. An example of such a murine myeloma cell line is P3X63AgU.1 described in Example 2 below. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against DR4.

Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding

assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

5 After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

10 The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

15 The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

30 As described in the Examples below, various anti-DR4 monoclonal antibodies have been identified and prepared. Certain of those antibodies, referred to as 4E7.24.3, 4H6.17.8, 1H5.25.9, 4G7.18.8, and 5G11.17.1 herein, have been deposited with ATCC. In one embodiment, the monoclonal antibodies of the invention will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) referred to above which have been deposited with ATCC. The term "biological characteristics" is used to refer to the *in vitro* and/or *in vivo* activities or properties of the monoclonal

antibody, such as the ability to specifically bind to DR4 or to block, induce or enhance DR4 activation (or DR4-related activities). As disclosed in the present specification (see Figure 6), the monoclonal antibody 4E7.24.3 is characterized as specifically binding to DR4 (and having some cross reactivity to Apo-2, DcR1 or DcR2), capable of inducing apoptosis, and not capable of blocking DR4. The monoclonal antibody 4H6.17.8 is characterized as specifically binding to DR4 (and having some cross-reactivity to Apo-2, DcR1 or DcR2), capable of inducing apoptosis, and capable of blocking Apo-2 ligand binding to DR4. The properties and activities of the 1H5.25.9, 4G7.18.8 and 5G11.17.1 antibodies are described in the Examples below (and also referred to in Fig. 17). Optionally, the monoclonal antibodies of the present invention will bind to the same epitope(s) as the 4E7.24.3, 4H6.17.8, 1H5.25.9, 4G7.18.8, and/or 5G11.17.1 antibodies disclosed herein. This can be determined by conducting various assays, such as described herein and in the Examples. For instance, to determine whether a monoclonal antibody has the same specificity as the DR4 antibodies specifically referred to herein, one can compare its activity in DR4 blocking assays or apoptosis induction assays, such as those described in the Examples below.

Further preferred antibodies of the invention include "cross-linked" DR4 antibodies. The term "cross-linked" as used herein refers to binding of at least two IgG molecules together to form one (or single) molecule. The DR4 antibodies may be cross-linked using various linker molecules, preferably the DR4 antibodies are cross-linked using an anti-IgG molecule, complement, chemical modification or molecular engineering. It is appreciated by those skilled in the art that complement has a relatively high affinity to antibody molecules once the antibodies bind to cell surface membrane. Accordingly, it is believed that complement may be used as a cross-linking molecule to link two or more anti-DR4 antibodies bound to cell surface membrane. Among the various murine Ig isotypes, IgM, IgG2a and IgG2b (such as the 1H5, 4G7, and 5G11 antibodies) are known to fix complement. The antibodies described in the Examples below, belonging to the murine IgG2 classes, were thus tested for apoptotic activity in the presence of rabbit complement. The apoptotic activity of the cross-linked antibodies (which was comparable to Apo-2L) suggests that complement or IgG-Fc cross-linkers may be useful in inducing oligomerization of such DR4 antibodies for, e.g., apoptosis of cancer cells.

The antibodies of the invention may optionally comprise dimeric antibodies, as well as multivalent forms of antibodies. Those skilled in the art may construct such dimers or multivalent forms by techniques known in the art and using the DR4 antibodies herein.

The antibodies of the invention may also comprise monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For

example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking.

Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an F(ab')₂ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH₁) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH₁ domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

Single chain Fv fragments may also be produced, such as described in Iliades et al., FEBS Letters, 409:437-441 (1997). Coupling of such single chain fragments using various linkers is described in Kortt et al., Protein Engineering, 10:423-433 (1997).

In addition to the antibodies described above, it is contemplated that chimeric or hybrid antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

3. Humanized Antibodies

The DR4 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences

of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296-2308 (1993); Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987)]. Another method uses a particular framework

derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285-4289 (1992); Presta et al., J. Immunol., 151:2623-2632 (1993)].

5 It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-2555 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggemann et al., Year in Immuno., 7:33-40 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381-388 (1991); Marks et al., J. Mol. Biol., 222:581-597 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77-96 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the DR4, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

6. Triabodies

Triabodies are also within the scope of the invention. Such antibodies are described for instance in Iliades et al., supra and Kortt et al., supra.

5 B. Uses for DR4 Antibodies

The DR4 antibodies of the invention have various utilities. For example, DR4 agonistic antibodies may be employed in methods for treating pathological conditions in mammals such as cancer. Diagnosis of such conditions are within the routine skill of the medical practitioner or
10 clinician. In the methods, the DR4 antibody, preferably an agonistic antibody, is administered to a mammal, alone or in combination with still other therapeutic agents or techniques.

The antibody is preferably administered to the mammal in a carrier; preferably a pharmaceutically-acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the carrier include saline, Ringer's solution and dextrose solution. The pH of the solution is preferably
15 from about 5 to about 8, and more preferably from about 7 to about 7.5. Further carriers include sustained release preparations such as semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of
20 administration and concentration of antibody being administered.

The antibody can be administered to the mammal by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular, intraportal), or by other methods such as infusion that ensure its delivery to the bloodstream in
25 an effective form. The antibody may also be administered by isolated perfusion techniques, such as isolated tissue perfusion, to exert local therapeutic effects. Local or intravenous injection is preferred.

Effective dosages and schedules for administering the antibody may be determined empirically, and making such determinations is within the skill in
35 the art. Those skilled in the art will understand that the dosage of antibody that must be administered will vary depending on, for example, the mammal which will receive the antibody, the route of administration, the particular type of antibody used and other drugs being administered to the mammal. Guidance in selecting appropriate doses for antibody is found in the literature on
40 therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies,

Ferrone et al., eds., Nokes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

The antibody may also be administered to the mammal in combination with effective amounts of one or more other therapeutic agents. The one or more other therapeutic agents or therapies may include, but are not limited to, chemotherapy, radiation therapy, immunoadjuvants, and cytokines. Other agents known to induce apoptosis in mammalian cells may also be employed, and such agents include TNF-alpha, TNF-beta, CD30 ligand, 4-1BB ligand and Apo-2 ligand.

Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, etoposide, camptothecin, Leucovorin, Cytosine arabinoside, Cyclophosphamide, Thiotepa, Busulfan, Cytosine, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. Preparation and dosing schedules for such chemotherapy may be used according to manufacturer's instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992).

The chemotherapy is preferably administered in a pharmaceutically-acceptable carrier, such as those described above. The mode of administration of the chemotherapy may be the same as employed for the DR4 antibody or it may be administered to the mammal via a different mode. For example, the DR4 antibody may be injected while the chemotherapy is administered orally to the mammal.

Radiation therapy can be administered to the mammal according to protocols commonly employed in the art and known to the skilled artisan. Such therapy may include cesium, iridium, iodine or cobalt radiation. The radiation therapy may be whole body radiation, or may be directed locally to a specific site or tissue in or on the body. Typically, radiation therapy is administered in pulses over a period of time from about 1 to about 2 weeks. The radiation therapy may, however, be administered over longer periods of time. Optionally, the radiation therapy may be administered as a single dose or as multiple, sequential doses.

The antibody may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of antibody and therapeutic agent depend, for example, on what type of drugs are used, the pathological condition being treated, and the scheduling and routes of administration but would generally be less than if each were used individually.

Following administration of antibody to the mammal, the mammal's physiological condition can be monitored in various ways well known to the skilled practitioner.

It is contemplated that the blocking DR4 antibodies may also be used in therapy. For example, a blocking DR4 antibody could be administered to a mammal (such as described above) to block receptor binding to Apo-2L, thus increasing the bioavailability of Apo-2L administered during Apo-2L therapy to induce apoptosis in cancer cells.

In another embodiment of the invention, methods for employing the antibody in diagnostic assays are provided. For instance, the antibodies may be employed in diagnostic assays to detect expression or overexpression of DR4 in specific cells and tissues. Various diagnostic assay techniques known in the art may be used, such as *in vivo* imaging assays, *in vitro* competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014-1021 (1974); Pain et al., J. Immunol. Meth., 40:219-230 (1981); and Nygren, J. Histochem. and Cytochem., 30:407-412 (1982).

DR4 antibodies also are useful for the affinity purification of DR4 from recombinant cell culture or natural sources. In this process, the antibodies against DR4 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the DR4 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the DR4, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the DR4 from the antibody.

In a further embodiment of the invention, there are provided articles of manufacture and kits containing materials useful for treating pathological conditions or detecting or purifying DR4. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of

materials such as glass or plastic. The container holds a composition having an active agent which is effective for treating pathological conditions or for detecting or purifying DR4. The active agent in the composition is a DR4 antibody and preferably, comprises monoclonal antibodies specific for DR4. The label on the container indicates that the composition is used for treating pathological conditions or detecting or purifying DR4, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention comprises the container described above and a second container comprising a buffer. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

EXAMPLE 1

Expression of DR4 ECD as an Immunoadhesin

A soluble DR4 ECD immunoadhesin construct was prepared. A mature DR4 ECD sequence (amino acids 1-218 shown in Fig. 1) was cloned into a pCMV-1 Flag vector (Kodak) downstream of the Flag signal sequence and fused to the CH1, hinge and Fc region of human immunoglobulin G₁ heavy chain as described previously [Aruffo et al., Cell, 61:1303-1313 (1990)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

EXAMPLE 2

Preparation of Monoclonal Antibodies Specific for DR4

Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5 µg/50 µl of a DR4 ECD immunoadhesin protein (as described in Example 1 above) (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals.

Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the DR4 ECD immunoadhesin protein (described in Example 1).

In the ELISA, 96-well microtiter plates (Maxisorp; Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 200 µl of 2.0% bovine serum albumin in PBS and incubated at room temperature for 1 hour. The plates were then washed again three times with wash buffer.

After the washing step, 50 µl of 0.4 µg/ml DR4 ECD immunoadhesin protein in assay buffer was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with wash buffer.

Following the wash steps, 100 µl of the hybridoma supernatants or Protein G-sepharose column purified antibody (10 µg/ml) was added to designated wells. 100 µl of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50 µl HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20 in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50 µl of substrate (TMB Microwell Peroxidase Substrate; Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50 µl of TMB 1-Component Stop Solution (Diethyl Glycol; Kirkegaard &

Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Hybridoma supernatants initially screened in the ELISA were considered for their ability to bind to DR4-IgG but not to CD4-IgG. The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing DR4; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25 μ l of cells suspended (at 4×10^6 cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02% NaN_3) were added to U-bottom microtiter wells, mixed with 100 μ l of culture supernatant or purified antibody (10 μ g/ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100 μ l FITC-conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150 μ l of cell sorter buffer and then analyzed by FACScan (Becton Dickinson, Mountain View, CA).

Figure 2 shows the FACS staining of 9D cells. Two particular antibodies, 4E7.24.3 and 4H6.17.8, recognized the DR4 receptor on the 9D cells.

EXAMPLE 3

Assay for Ability of DR4 Antibodies to Agonistically induce Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 2 above) were tested for activity to induce DR4 mediated 9D cell apoptosis. The 9D cells (5×10^5 cells/0.5ml) were incubated with 5 μ g of DR4 mAbs (4E7.24.3 or 4H6.17.8; see Example 2 above) or IgG control antibodies in 200 μ l complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C with or without 10 μ g of goat anti-mouse IgG Fc antibody (ICN Pharmaceuticals) in 300 μ l of complete RPMI. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO_2 . The cells were then harvested and washed once with PBS. The apoptosis of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200 μ l binding buffer. Ten μ l of annexin-V-FITC (1 μ g/ml) and 10 μ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

As shown in Figure 3, both DR4 antibodies (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity of both DR4 antibodies, however, was enhanced by DR4 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (See Figure 4). This enhanced apoptosis (Figure 4) by both DR4 antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells.

EXAMPLE 4

Assay for DR4 Antibody Ability to Block Apo-2L-induced 9D Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 2 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis.

The 9D cells (5×10^5 cells/0.5 ml) were suspended in complete RPMI media (RPMI plus 10% FCS, glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate) and preincubated with serially diluted DR4 antibody (4H6.17.8) and/or an Apo-2 antibody (mAb 3F11, ATCC No. HB-12456) in individual Falcon 2052 tubes. The tubes containing the cells were incubated on ice for 15 minutes and then about 0.5 ml of Apo-2L ($1 \mu\text{g/ml}$; soluble His-tagged Apo-2L prepared as described in WO 97/25428) was suspended into complete RPMI media, added to the tubes containing the 9D cells and antibody, and then incubated overnight at 37°C and in the presence of 7% CO_2 . The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). Specifically, the cells were washed in PBS and resuspended in $200 \mu\text{l}$ binding buffer. Ten ml of annexin-V-FITC ($1 \mu\text{g/ml}$) and $10 \mu\text{l}$ of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

The results are shown in Figure 5. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either DR4 or the receptor referred to as Apo-2. Thus, to detect any blocking activity of the DR4 antibodies, the interaction between Apo-2 and Apo-2L needed to be blocked. In combination with the blocking anti-Apo-2 antibody, 3F11, the DR4 antibody 4H6.17.8 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activity of the DR4 antibodies alone, as shown in Figure 5. Accordingly, it is believed that 4H6.17.8 is a blocking DR4 antibody.

EXAMPLE 5

Antibody Isotyping

The isotypes of the 4H6.17.8 and 4E7.24.3 antibodies (as described above) were determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C . The plates were then washed with wash buffer (as described in Example 2 above). The wells in

the microtiter plates were then blocked with 200 μ l of 2% bovine serum albumin and incubated at room temperature for one hour. The plates were washed again three times with wash buffer.

Next, 100 μ l of 5 μ g/ml of purified DR4 antibodies or 100 μ l of the hybridoma culture supernatant was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 μ l HRP-conjugated goat anti-mouse IgG (as described above) was added to each well. The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

The isotyping analysis showed that the 4E7.24.3 and 4H6.17.8 antibodies are IgG1 antibodies.

EXAMPLE 6

ELISA Assay to Test Binding of DR4 Antibodies to Other Apo-2L Receptors

An ELISA was conducted to determine if the two DR4 antibodies described in Example 2 were able to bind other known Apo-2L receptors beside DR4. Specifically, the DR4 antibodies were tested for binding to Apo-2 [see, e.g., Sheridan et al., *Science*, 277:818-821 (1997)], DcR1 [Sheridan et al., *supra*], and DcR2 [Marsters et al., *Curr. Biol.*, al., 7:1003-1006 (1997)]. The ELISA was performed essentially as described in Example 2 above.

The results are shown in Figure 6. The DR4 antibodies 4E7.24.3 and 4H6.17.8 bound to DR4, and showed some cross-reactivity to Apo-2, DcR1 or DcR2.

EXAMPLE 7

Preparation of Monoclonal Antibodies Specific for DR4

Monoclonal antibodies to DR4 were produced essentially as described in Example 2. Using the capture ELISA described in Example 2, additional anti-DR4 antibodies, referred to as 1H5.24.9, 1H8.17.5, 3G1.17.2, 4G7.18.8, 4G10.20.6 and 5G11.17.1 were identified. (See Table in Figure 17) Further analysis by FACS (using the technique described in Example 2) confirmed binding of these antibodies to 9D cells expressing DR4 (data not shown).

EXAMPLE 8

Antibody Isotyping

The isotypes of the 1H5.24.9, 1H8.17.5, 3G1.17.2, 4G7.18.8, 4G10.20.6 and 5G11.17.1 anti-DR4 antibodies (described in Example 7) were determined essentially as described in Example 5.

The isotyping analysis showed that the 1H8.17.5, 3G1.17.2 and 4H10.20.6 are IgG1 antibodies. Anti-DR4 antibodies 1H5.24.9 and 4G7.18.8 are IgG2a antibodies, and antibody 5G11.17.1 is an IgG2b antibody.

5

EXAMPLE 9

Determination of Monoclonal Antibody Affinities

The equilibrium dissociation and association constant rates of various DR4 antibodies (described in the Examples above) were determined using KinExA™, an automated immunoassay system (Sapidyne Instruments, Inc., Boise, ID), as described with a modification by Blake et al., Journal of Biological Chemistry, 271:27677-685 (1996); and Craig et al., Journal of Molecular Biology, 281:183-201 (1998). Briefly, 1.0 ml of anti-human IgG agarose beads (56 μ m, Sigma, St. Louis, MO) were coated with 20 μ g of DR4-IgG (described in Example 1) in PBS by gentle mixing at room temperature for 1 hour. After washing with PBS, non-specific binding sites were blocked by incubating with 10% human serum in PBS for 1 hour at room temperature.

A bead pack (~4 mm high) was created in the observation flow cell by the KinExA™ instrument. The blocked beads were diluted into 30 ml of assay buffer (0.01% BSA/PBS). The diluted beads (550 μ l) were next drawn through the flow cell with a 20 μ m screen and washed with 1 ml of running buffer (0.01% BSA; 0.05% Tween 20 in PBS). The beads were then disrupted gently with a brief backflush of running buffer, followed by a 20 second setting period to create a uniform and reproducible bead pack. For equilibrium measurements, the selected DR4 antibodies (5 ng/ml in 0.01 % BSA/PBS) were mixed with a serial dilution of DR4-IgG (starting from 2.5 nM to 5.0 pM) and were incubated at room temperature for 2 hours. Once equilibrium was reached, 4.5 ml of this mixture was drawn through the beads, followed by 250 μ l of running buffer to wash out the unbound antibodies. The primary antibodies bound to beads were detected by 1.5 ml of phycoerythrin labeled goat anti-mouse IgG (Jackson Immunoresearch). Unbound labeled material was removed by drawing 4.5 ml of 0.5 M NaCl through the bead pack over a 3 minute period. The equilibrium constant was calculated using the software provided by the manufacturer (Sapidyne, Inc.).

The affinity determinations for the DR4 antibodies are shown in Figure 7. Affinity determinations for immunoadhesin constructs of the DR4 and DR5 receptors for Apo-2L, and for the DR5 antibody, 3F11, for an Ig construct of DR5, are shown for comparison. The affinities (Kd-1) of the 4E7.24.3, 4H6.17.8 and 5G11 antibodies were 2 pM, 5 pM, and 22 pM, respectively,

demonstrating that these monoclonal antibodies have strong binding affinities to DR4-IgG.

EXAMPLE 10

Apoptosis Assay of Lymphoid Tumor Cells Using DR4 Antibodies

Apoptosis of human 9D B lymphoid tumor cells induced by anti-DR4 monoclonal antibodies was examined.

Human 9D cells (5×10^5) were suspended in 100 microliter complete RPMI medium (RPMI plus 10% FCS, glutamine, nonessential amino acids, penicillin, streptomycin and sodium pyruvate) and added to 24 well macro-titer wells (5×10^5 cells/0.5 ml/well). 100 microliter of 10 microgram/ml of purified DR4 antibody or 100 microliter of culture supernatant and then added into the wells containing 9D cells. The cells were then incubated overnight at 37° C in the presence of 7% CO₂.

At the end of the incubation, cells were washed once with PBS. The washed cells were resuspended in 200 microliter binding buffer (Clontech) and 10 microliter of FITC-Annexin V (Clontech) and 10 microliter of propidium iodide were added to the cells. [See, Moore et al., Cell Biol., 57:265 (1998)]. After incubation for 15 minutes in the dark, the cells were analyzed by FACSscan.

The results are shown in Figure 8A. The graphs in Figure 8A show that the 1H5, 4G7, and 5G11 antibodies by themselves induced some (weak) apoptosis in the 9D cells, but the apoptotic activity of each antibody was markedly increased when these monoclonal antibodies were cross-linked by either goat anti-mouse IgG-Fc or complement (as described in Example 11 below).

EXAMPLE 11

Apoptosis Assay of 9D Cells Using Cross-linked DR4 Antibodies

The apoptotic activity of cross-linked DR4 antibodies on 9D cells was also examined. The 9D cells (5×10^5) were suspended in 100 microliter complete RPMI medium (RPMI plus 10% FCS, glutamine, nonessential amino acids, penicillin, streptomycin and sodium pyruvate) and incubated with 1 microgram of DR4 antibody/100 microliter on ice for 15 minutes. The cells were incubated with a 1:10 final dilution of rabbit complement (Cedar Lane) or 100 microgram/ml of goat anti-mouse IgG-Fc (Cappel Laboratories) in 300 microliter complete medium overnight at 37° C in the presence of 7% CO₂.

At the end of the incubation, cells were washed once with PBS and suspended in 200 microliter of binding buffer (Clontech). Next, 10 microliter of FITC-Annexin V (Clontech) and 10 microliter of propidium iodide were added

to the cells. [See, Moore et al., Cell Biol., 57:265 (1998)]. After incubation for 15 minutes in the dark, the cells were analyzed by FACScan.

The results are shown in Figures 8A and 8B. The results show that the 4G7.17.8, 5G11.17.1 and 1H5.24.9 anti-DR4 antibodies induced apoptosis of 9D cells when cross-linked with goat anti-mouse IgG or rabbit complement, although the degree of apoptosis induced using complement as a linker was not as potent as compared to the use of the goat anti-mouse IgG-Fc linker. However, the apoptotic activity of the cross-linked DR4 antibodies (at concentrations of about 1-2 microgram/ml) was comparable to the apoptotic activity of Apo-2L at similar concentrations.

EXAMPLE 12

Apoptosis Assay of Human Lung and Colon Tumor Cell Lines

The apoptotic activities of the monoclonal antibodies were further examined in assays to determine the cell viability of cancer cells after treatment with the antibodies or Apo-2L.

SKMES-1 cells (human lung tumor cell line; ATCC) and HCT-116 cells (human colon tumor cell line; ATCC) were seeded at 4×10^4 cells/well in complete high glucose 50:50 medium supplemented with glutamine, penicillin and streptomycin, in tissue culture plates and allowed to attach overnight at 37°C. The media was then removed from the wells, and 0.1 ml of antibody (anti-DR4 antibodies diluted 0.001-10 microgram/ml in complete medium) was added to selected wells. Control wells without antibody received a media change with or without Apo-2L. The plates were then incubated for 1 hour at room temperature.

The culture supernatant was removed from the wells containing the test antibodies, and 10 microgram/ml goat anti-mouse IgG-Fc (Cappel Laboratories) or rabbit complement (Cedar Lane; diluted in medium to 1:10) was added to the wells. Media was changed in the control wells. The plates were incubated overnight at 37°C. As a control, Apo-2L (as described in Example 4) (in potassium phosphate buffer, pH 7.0) was diluted to 2 microgram/ml. 0.1 ml of the diluted Apo-2L solution was added to selected wells, and then serial three-fold dilutions were carried down the plate.

Culture supernatants were then removed from the wells by aspiration, and the plates were flooded with 0.5% crystal violet in methanol solution. After 15 minutes, the crystal violet solution was removed by flooding the plates with running tap water. The plates were then allowed to dry overnight.

Absorbance was read on an SLT 340 ATC plate reader (Salzburg, Austria) at 540 nm. The data was analyzed using an Excel macro and 4p-fit. The results illustrating the activity of the DR4 antibodies on SKMES cells are shown in Figures 9 and 10. Figures 9 and 10A show that the 1H8.17.5,

4E7.24.3, 4G7.17.8, 4H6.17.8, 4G10.20.6, and 5G11.17.1 antibodies induced cell death of the SKMES cells when the cells were incubated with the respective antibodies plus goat anti-mouse IgG Fc. In contrast, the 3G1.17.2 antibody did not induce cell death in the cells, even in the presence of the IgG Fc cross-linker. Figure 10B illustrates the apoptotic activity of the 4G7 (IgG2a isotype) and 5G11 (IgG2b isotype) antibodies on the SKMES cells in the presence of rabbit complement.

The results illustrated in Figure 11 show the activity of the DR4 antibodies on the HCT116 colon cancer cells. The IgG2 isotype DR4 antibodies, 4G7 and 5G11, induced apoptosis in the colon cancer cells in the presence of IgG Fc or complement. The DR4 antibody, 4E7 (IgG1 isotype), did not induce apoptosis in the presence of complement, although the antibody did demonstrate potent apoptotic activity in the presence of goat anti-mouse IgG Fc.

EXAMPLE 13

ELISA Assay to Test Binding of DR4 Antibodies to Other Apo-2L Receptors

An ELISA assay was conducted (as described in Examples 2 and 6) to determine binding of the DR4 antibodies to other known Apo-2L receptors, beside DR4.

The 5G11.17.1 antibody bound to DR4 and Apo-2, and showed some (weak) cross-reactivity to DcR1 and DcR2. The 4G10.20.6 antibody bound to DR4 and showed some (weak) cross-reactivity to Apo-2. The other antibodies, 1H8.17.5, 4G7.18.8, 1H5.24.9, and 3G1.17.2, bound to DR4 but not to any of the Apo-2, DcR1, or DcR2 receptors.

EXAMPLE 14

poly ADP-ribose polymerase (PARP) assay

A PARP assay was conducted to determine whether the activity induced by the IgG2 anti-DR antibodies was achieved by apoptosis or by conventional complement lysis.

9D cells (5×10^5 cells in 100 μ l of complete medium (described in Example 11) were incubated with 100 μ l of antibody (4G7 or 5G11) (1 mg/ml) for 15 minutes on ice. Then, 300 μ l of Rabbit Complement (Cedar Lane; diluted with 1.0 ml of cold distilled water followed by the addition of 2.0 ml of media) was added to the cells. The cells were then incubated overnight at 37° C. At the end of the incubation, the cells were microcentrifuged, harvested and washed once in cell wash buffer (50mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM CaCl_2 , 1 mM MgCl_2). The cell pellets were then lysed with 50 μ l of cell lysis buffer (cell wash buffer plus 1% NP40) containing protease inhibitors, incubated on ice for 30 minutes, and then spun at 13,000rpm for 10 minutes.

The cell lysate was mixed with an equal volume of 2X SDS reducing buffer. After boiling 2 minutes, proteins were separated onto a 7.5% SDS PAGE gel and transferred to immunoblot PVDF membranes (Gelman). After blocking the nonspecific binding sites with blocking buffer (Boehringer Mannheim), poly-
5 (ADP-ribose)-polymerase was detected using HRP-rabbit anti-poly(ADP-ribose)-polymerase (Boehringer Mannheim). This antibody will detect the intact (116Kd) as well as degraded (85Kd) PARP which is generated as an early step of apoptosis. Bound anti-HRP-rabbit anti-poly-(ADP-ribose)-polymerase was detected using chemiluminescent immunoassay signal reagents according to
10 manufacturer instructions (Amersham, Arlington Heights, IL).

The results are shown in Figure 12. The cells treated with either 4G7 or 5G11 plus complement demonstrated the presence of cleaved 85 Kd PARP, indicating that the mechanism of the 9D cell death induced by the respective antibodies was due to apoptosis. When the complement added to the assay was
15 heat inactivated by incubating for 30 minutes at 56°C, the 85 Kd cleaved fragment of PARP was not detectable. The results suggest that the complement in the rabbit serum induced the oligomerization of the anti-DR4 antibodies bound to the cells, resulting in the apoptosis of the 9D cells.

EXAMPLE 15

In vivo Activity of DR4 Antibodies

Since the class IgG2 DR4 antibodies induced apoptosis in the presence of complement (described in the above Examples), an *in vivo* assay was conducted to determine if these antibodies may be able to induce apoptosis of tumor
25 cells *in vivo* in the presence of native complement molecules present in the animal.

HCT116 cells (human colon tumor cell line; ATCC) or Colo205 cells (human colon tumor cell line; ATCC) were grown in high glucose F-12:DMEM (50:50)
30 medium supplemented with 10% FCS, 2 mM glutamine, 100 µg/ml of penicillin, and 100 µg/ml streptomycin. The cells were harvested after treating with cell dissociation medium (Sigma, IAC) for 5 minutes. After washing in PBS, the tumor cells were resuspended in PBS at a concentration of 3×10^7 cells/ml.

Nude mice were injected with $3-5 \times 10^6$ cells subcutaneously in the
35 dorsal area in a volume of 0.1 ml. When the tumor size in the HCT116 tumor bearing animals became a desired size, the mice were injected i.p. with 100 µg of monomeric anti-DR4 antibody in PBS three times per week, and the tumor sizes were measured three times/week. The Colo205 tumor bearing animals were injected i.p. with varying concentrations of the DR4 antibodies, 4G7 and 4H6
40 (as shown in Figures 15 and 16). At the end of the experiment examining the

HCT116 tumors, the mice were sacrificed, and the weight of each tumor was determined.

The results illustrated in Figures 13 and 14 show that both 4G7 and 5G11 inhibited the growth of HCT116 tumors. There was approximately 35-40% and 50% growth inhibition of HCT116 tumors after treatment with antibodies 5G11 and 4G7, respectively.

The results illustrated in Figures 15 and 16 show that both 4G7 and 4H6 inhibited growth of Colo205 tumors. Fig. 15 illustrates that the antibody treatment was more effective when the size of the tumors were smaller. Fig. 16 shows that of the mice treated with 25-200 microgram of 4G7 (injected three times per week), the mice receiving the 50 microgram doses of 4G7 achieved the maximum inhibition (70%) of Colo205 tumor growth. The 4H6 antibody shrunk the Colo205 tumor growth to near zero after treatment for 10 days. At the end of 10 days treatment of 4H6 (100 microgram/injection), 4/8 mice showed no Colo205 tumor growth (data not shown).

The results suggest that these DR4 antibodies induced apoptosis by oligomerization with native complement present in the animal. It is believed that anti-DR4 antibodies of human Ig isotypes such as IgG1, IgG2, or IgG3 (which can fix complement), may similarly be capable of cross-linking using complement and inducing apoptosis.

* * * *

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
4E7.24.3	HB-12454	Jan. 13, 1998
4H6.17.8	HB-12455	Jan. 13, 1998
1H5.25.9	HB-12695	April 1, 1999
4G7.18.8	_____	May 21, 1999
5G11.17.1	HB-12694	April 1, 1999

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and

ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC '122 and the Commissioner's rules pursuant thereto (including 37 CFR '1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

1. An isolated antibody which specifically binds to DR4.
2. The antibody of Claim 1 which is an agonist antibody.
3. The antibody of Claim 1 which is a blocking antibody.
4. The antibody of Claim 1 which is a monoclonal antibody.
5. The antibody of Claim 1 which is a murine antibody.
6. The antibody of Claim 1 which is a humanized antibody.
7. The antibody of claim 1 which is a chimeric antibody.
8. The antibody of claim 1 which is a monomeric antibody.
9. The antibody of claim 1 which is a multivalent antibody.
10. A hybridoma cell line which produces the antibody of Claim 4.
11. The antibody of Claim 4 having the same biological characteristics of (1) the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12695; (2) the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12694; or (3) the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC ____.
12. The antibody of Claim 4 wherein the antibody binds to the same epitope as (1) the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12695 binds; (2) the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited under the American Type Culture Collection Accession Number ATCC HB-12694 binds; or (3) the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC ____.
13. The hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12695.
14. The hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12694.
15. The hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC ____.
16. The monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12695.
17. The monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12694.
18. The monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC ____.

19. An isolated nucleic acid comprising DNA encoding the DR4 antibody of Claim 1.
20. A composition comprising the antibody of Claim 1 and a carrier.
21. The composition of claim 20 wherein said carrier is a pharmaceutically acceptable carrier.
22. A method of inducing apoptosis in mammalian cancer cells comprising exposing mammalian cells to an effective amount of a DR4 antibody.
23. An article of manufacture, comprising a container and a composition contained within said container, wherein the composition includes a DR4 antibody.
24. The article of manufacture of claim 23 further comprising instructions for using the DR4 antibody *in vivo* or *ex vivo*.
25. A dimeric molecule comprising a DR4 antibody.
26. The dimeric molecule of claim 25 comprising a DR4 antibody linked to a complement molecule.
27. A method of treating cancer in a mammal, comprising administering to said mammal an effective amount of DR4 antibody.
28. The method of claim 27 wherein said DR4 antibody is linked to a heterologous immunoglobulin.
29. The method of claim 27 wherein said DR4 antibody is linked to a complement molecule.
30. The method of claim 27 wherein said cancer is colon cancer.
31. The method of claim 27 wherein said cancer is lung cancer.
32. The method of claim 27 wherein chemotherapy is also administered to said mammal.

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
--	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	------	---

5

1 ATGGGGCCAC CACCAGCTAG AGTACATCTA GGTGGGTTCC TGGCAGTGAC TCGGATTCG GCGAGCGCAG CCAGTGGGAC AGAGGCAGCC GCGGGCCACAC
TACCGCGGTG GTGGTCGATC TCATGTAGAT CCACGCNAGG ACCGTACATG AGGCTTAGG CCCTCGCGTC GCTCACCCCTG TCCTCGTCGG GCCTCGGTGTG
1 MetAlaProp roProAlaAr gValHisLeu GlyAlaPheL euAlaValTh rProAsnPro GlySerAlaA laseGlyTh rGluAlaAla AlaAlaThrPro
101 CCAGCAAAAGT GTGGGGCTCT TCCGGCGGGA GGATTGAACC ACGAGGCGGG GGCCGAGGAG CGCTCCCTAC CTCCATGGGA CAGCACGGAC CCAGTGCCCG
GGTCGTTTCA CACCCCGAGA AGGCGCCCT CCTAACTTGG TGCTCCGCC CCGGCTCCTC GCGAGGGATG GAGGTACCCT GTCTGCCTG GGTACCGGGC
35 SerLysVa lTrpGlySer SerAlaGlyA rgileGluPr oArgGlyGly oArgGlyA laLeuProTh rSerMetGly GlnHisGlyP roSerAlaArg
201 GGGCCGGGCA GGGCGGGCC CAGGACCCAG GCGGGCGGG GAAGCCAGCC CTGCGGCTCG GGTCCACAAG ACCTTCAAGT TTGTGCTCGT CCGGGTCTCTG
CCGGGGCCGT CCGCGCGGG GTCTGGGTC GTCCTGGGTC CTTCCGGTGG GAGCCGAGG CCAGGTGTTT TGGAAAGTTCA AACAGCAGCA GCGCCAGGAC
68 AlaArgAla GlyArgAlap roGlyProAr gProAlaArg GluAlaSerP roArgLeuAr gValHisLys ThrPheLysP heValValva lGlyValLeu
301 CTGCAGGTCTG TACCTAGCTC AGCTGCAACC ATCAAACTTC ATGATCAATC AATTGGCACA CAGCAATGGG AACATAGCCC TTTGGGAGAG TTGTGTCCAC
GACGTCCAGC ATGATCGAG TCGACGTTGG TAGTTTGAAG TACTAGTTAG TTAACCGTGT GTCGTTACCC TTGTATCGGG AAACCTCTC AACACAGGTG
101 LeuGlnValV alProSerSe rAlaAlaThr lLeLysLeuH iAspGlnSe rileGlyThr GlnGlnTrpG luHisSerPr oLeuGlyGlu LeuCysProPro
401 CAGGATCTCA TAGATCAGAA CGTCTGGAG CCTGTAACCG GTGCACAGAG GGTGTGGGTT ACACCAATGC TTCCAAACAAT TTGTTTGCTT GCCTCCCATG
GTCCTAGAGT ATCTAGTCTT GCAGGACCTC GGACATTGGC CACGTGTCTC CCACACCCAA TGTGTTTACG AAGGTTGTTA AACAAACGAA CCGAGGGTAC
135 GlySerHi sArgSerGlu ArgProGlyA laCysAsnAr gCysThrGlu GlyValGlyT yrThrAsnAl aSerAsnAsn LeuPheAlaC ysLeuProCys
501 TACAGCTTGT AATCAGATG AAGAAGAGAG AGTCCCTGC ACCACGACCA GGACACAGC ATGTCAGTGC AAACAGGAA CTTTCCGGAA TGACAAATTCT
ATGTGGAACA TTAGTCTAC TTCTTCTCTC TTCAGGGACG TGGTGCTGGT CCTTGTCG TACAGTCACG TTTGGTCCCTT GAAAGGCCCTT ACTGTTAAGA
168 ThrAlaCys LysSerAspG luGluGluAr gSerProCys ThrThrThra rAsnThral aCysGlnCys LysProGlyT hrPheArgAs naspsnSer
601 GCTGAGATGT GCCGGAAGTG CAGCACAGG TGCCCCAGAG GGATGGTCAA GGTCRAAGGAT TGTACGCCCTT GGAGTCACAT CGAGTGTGTG CACAAAGAAT
CGACTCTACA CGGCTTCC GTCGTCTCC ACGGGTCTC CCTACCAGT CCAGTTCTTA ACATGCGGGA CTTCACTGTA GCTCACACAG GTGTTTCTTA
201 AlaGluMetC ysArgLysCy sserThrGly CysProArgG lyMetVally svalLysasp CysThrProt rPserAspIl eGluCysval HisLysGluSer
701 CAGGCAATGG ACATAATATA TGGGTGATTT TGGTGTGAC TTTGGTTGTT CCGTTGCTGT TGGTGGCTGT GCTGATTGTC TGTGTTGCA TCGGCTCAGG
GTCCGTTACC TGTATTATAT ACCCACTAA ACCAACACTG ACCAACACAA GGCAACGACA ACCACCGACA CCACTAACAG ACAACACAGT AGCCGAGTCC
235 GlyAsnGl yHisAsnIle TrpValIleL euValValTh rLeuValVal ProLeuLeuL euValAlaVa lLeuIleVal CysCysCysI leGlySerGly
801 TTGTGGAGGG GACCCCAAGT GCATGGACAG GGTGTGTTTC TGGCGTTGG GTCTCCTACG AGGCCCTGGG GCTGAGGACA ATGCTACAA CGAGATTCTG
AACACCTCCC CTGGGGTTCA GTACCTGTG CACACACAAAG ACCGGGAACC CAGAGGATGC TCCCGGACCC CGACTCCTGT TACGAGTGT TCTTAAGAC
268 CysGlyGly AspProLysC ysMetAspAr gValCysPhe TrpArgLeuG lyLeuLeuAr gGlyProGly AlaGluAspA snAlaHisAs nGluIleLeu
901 AGCAACGCAG ACTCGCTGTC CACTTTCGTC TCTGAGCAGC AATGGAAG CCAGGAGCCG GCAGATTGA CAGGTGTCAC TGTCACAGTCC CCAGGGGAGG
TCGTTGCGTC TGAGCGACAG GTGAAGCAG AGACTCGTCG TTTACCTTTC GGTCTCGGC CGTCTAACT GTCCACAGTG ACATGTCAGG GGTCCCTCC
301 SerAsnAlaA spSerLeuSe rThrPheVal serGluGlnG lnMetGluse rGlnGluPro AlaAspLeuT hrGlyValTh rValGlnSer ProGlyGluAla
1001 CACAGTGTCT GCTGGGACCG GCAGAAGCTG AAGGTTCTCA GAGGAGGAG CTGCTGTTTC CAGCAAAATGG TGCTGACCCC ACTGAGACTC TGATGCTGT
GTGTCACAGA CGACCTGGC CGTCTTCGAC TTCCACAGAT CTCCTCTCC GACGACCAAG GTCGTTTACC ACGACTGGG TGACTCTGAG ACTACGACAA
335 GlnCysLe uLeuGlyPro AlaGluAlag luGlySerGl nArgArgArg LeuLeuValP roAlaAsnGl yAlaAspPro ThrGluThrL euMetLeuphe

Fig. 1

1101 CTTTGACAAG TTTGCAACA TCGTGGCTTT TCGATGTGAG TCGATGTGGT CAGAGCTGGT
 GAAACTGTTT AAACGTTTGT AGCAGCGGAAA ACTGAGGACCC CTTGGTGGAGT ACTCCGTCGA CCTGGAGTGC TTTTCTACTCT AGCTACACCA GTCTCGACCA
 368 PheAspLys PheAlaAsnI leValProPh eAspSerTrp AspGlnLeuM eArgGlnLe uAspLeuThr LysAsnGlnI leAspValva lArgAlaGly
 1201 ACAGCAGGCC CAGGGGATGC CTTGTATGCA ATGCTGATGA AATGGGTCAA CAAACTGGA CGGAACGCC T CGATCCACAC CCTGCTGGAT GCCTTGGAGA
 TGTGTCGGG GTCCCCCTACG GAACATACGT TACGACTACT TTACCCAGTT GTTTTGACCT GCCTTGGGA GCTAGGTGTG GGACGACCTA CGGAACCTCT
 401 ThrAlaGlyP roGlyAspAl aLeuTyrrAla MetLeuMetL ystrpValas nLysThrGly ArgAsnAlas erileHisTh rLeuLeuAsp AlaLeuGluArg
 1301 GGATGGAAGA GAGACATGCA AAAGAGAAGA TTCAGGACCT CTTGGTGGAC TCTGGAAGT TCATCTACTT AGAAGATGGC ACAGGCTCTG CCGTGTCTCTT
 CCTACCTTCT CTCTGTACGT TTTCTCTTCT AAGTCTGGA GAACCACTG AGACCTTTCA AGTAGATGAA TCTTCTACCG TGTCCGAGAC GGCACAGGAA
 435 MetGluGl uArgHisAla LysGluLysI leGlnAspLe uLeuValasp serGlyLysP heileTyrrLe uGluAspGly ThrGlySera laValSerLeu
 1401 GGACTGA
 CCTCACT
 468 GluOP*

Fig. 1 (cont.)

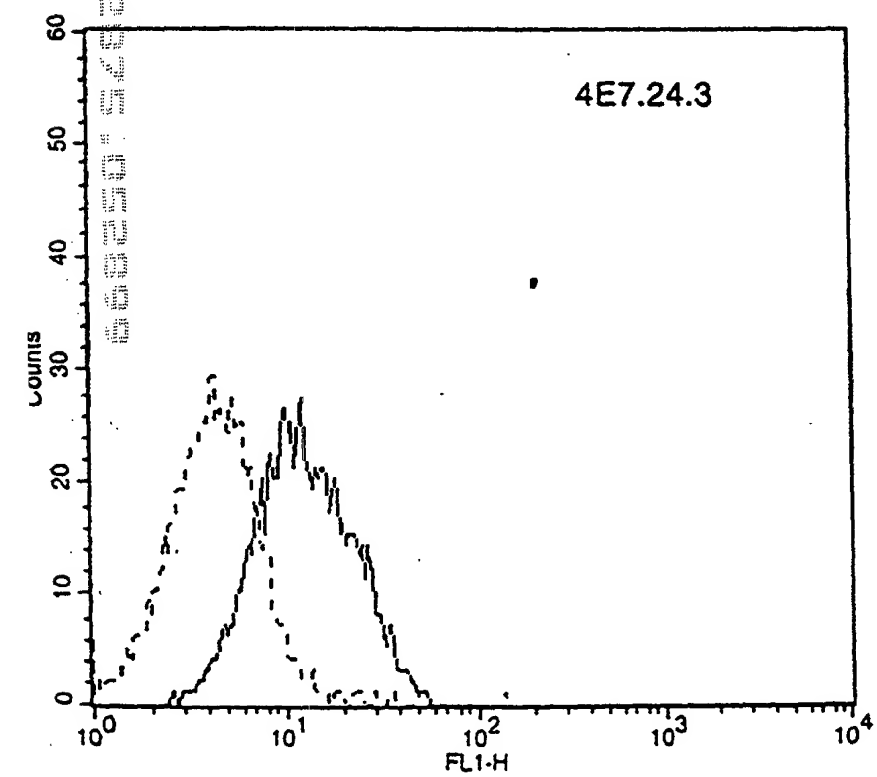
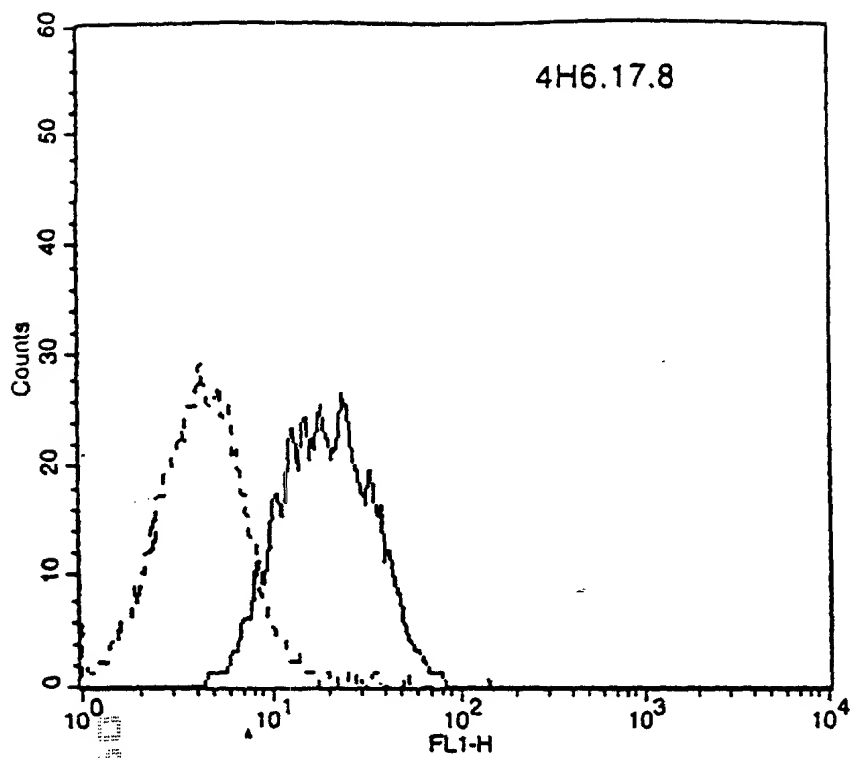


Fig. 2

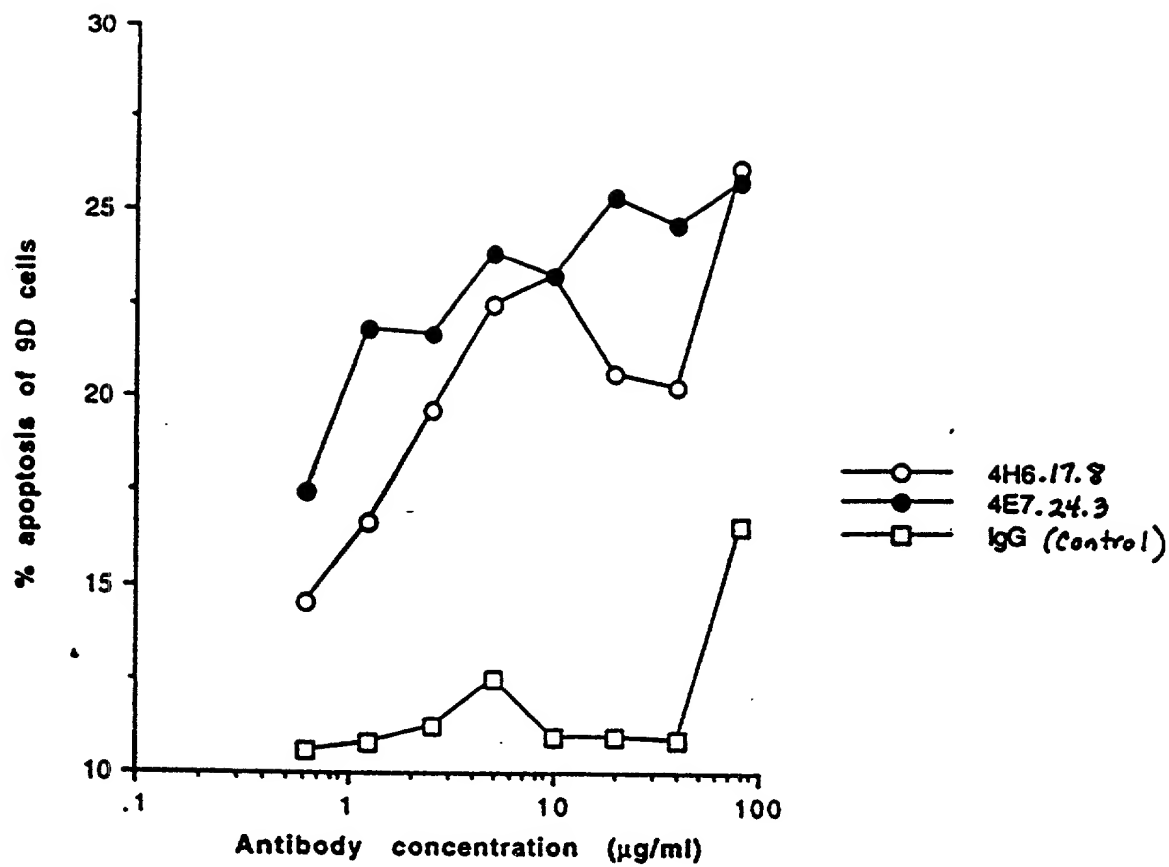


Fig. 3

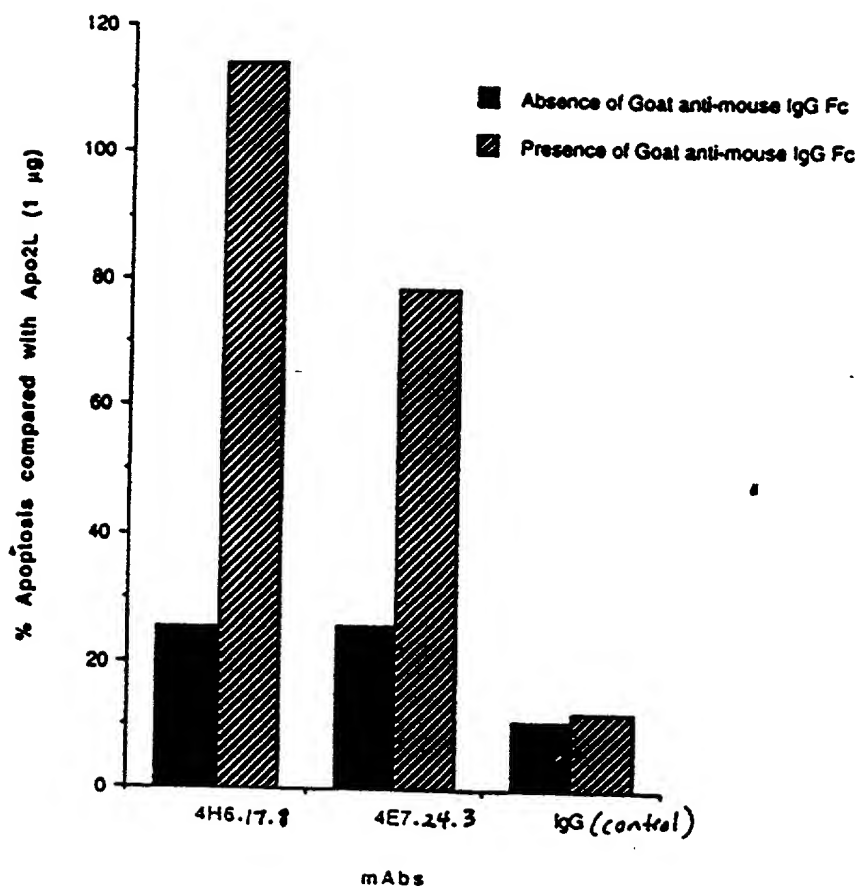


Fig. 4

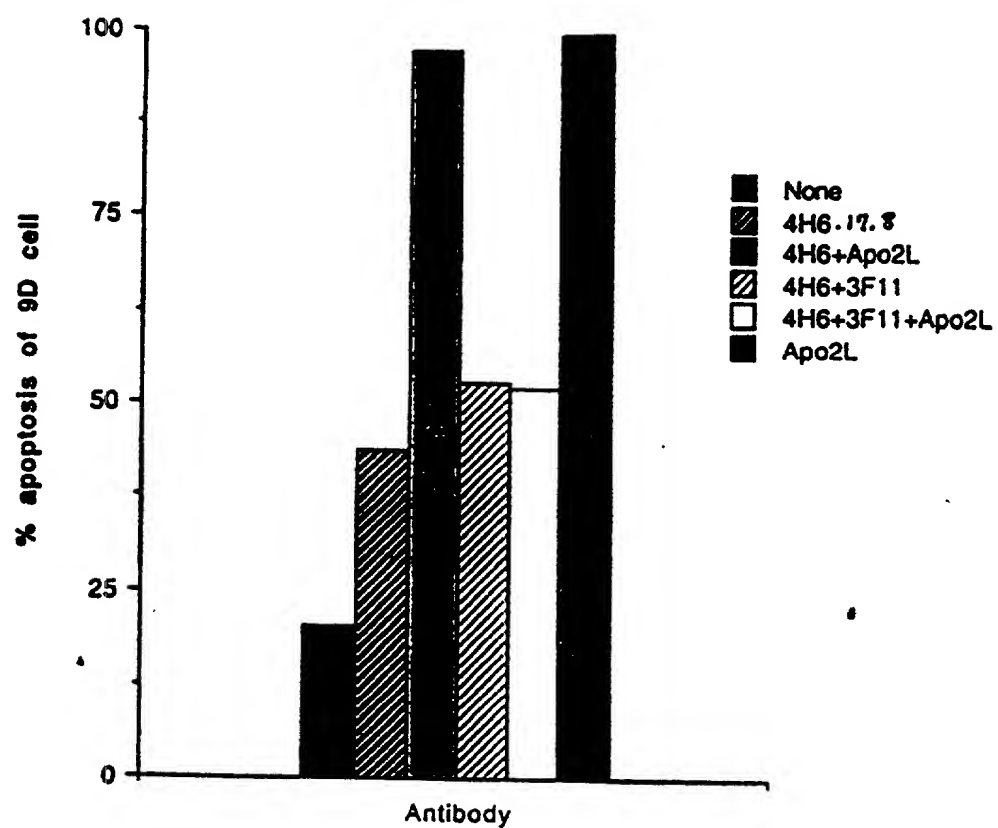
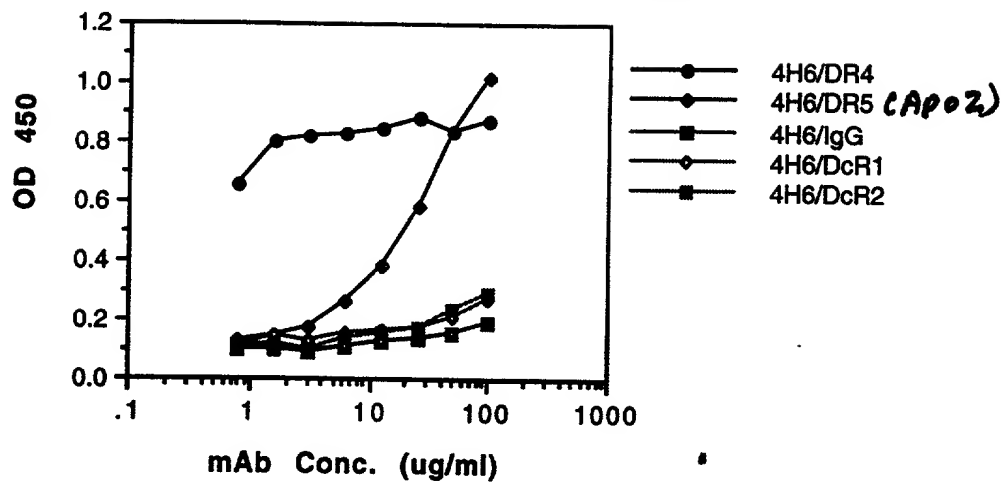


Fig. 5

4H6: Binding to receptors for Apo2L



4E7: Binding to Receptors for Apo2L

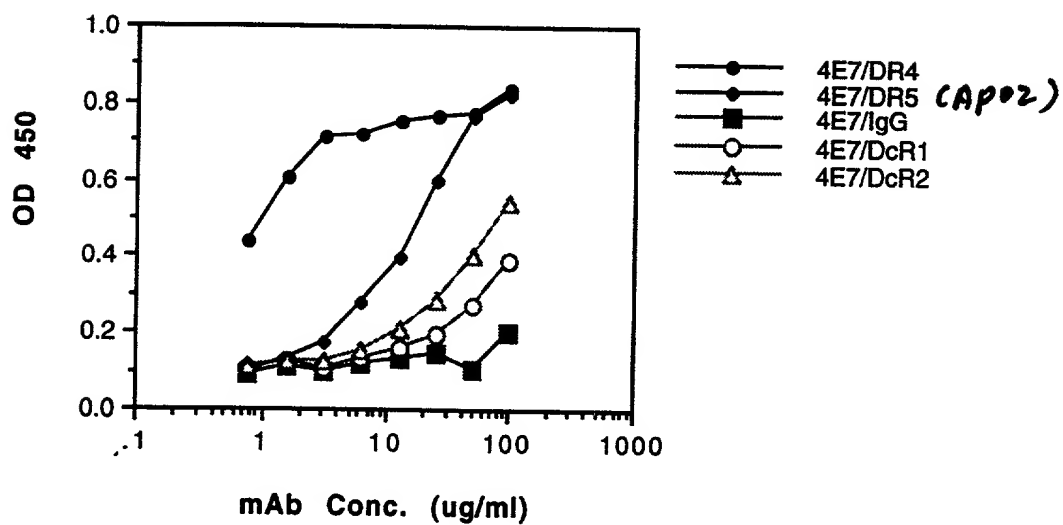


Fig. 6

Affinities of Apo2Rs and mAbs

		Affinity (pM)
DR4-IgG	to Apo2L	82
DR5-IgG	to Apo2L	1
mAb 4E7	to DR4-IgG	2
mAb 4H6	to “	5
mAb 5G11	to “	22
mAb 3F11	to DR5-IgG	20
mAb 3H3	to “	3

Affinities were determined using KinExA

Fig. 7

Apoptosis of 9D cells by anti-DR4 mAbs plus complements or goat anti-mouse Ig-FC

Percent Apoptosis compared to Apo2L

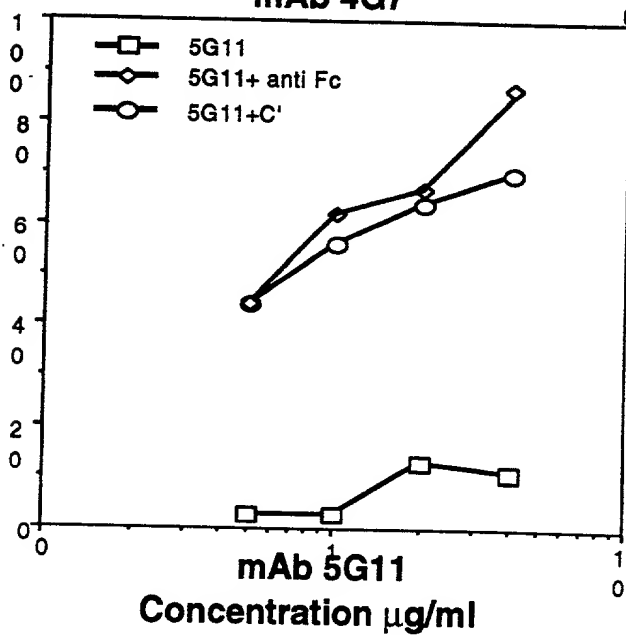
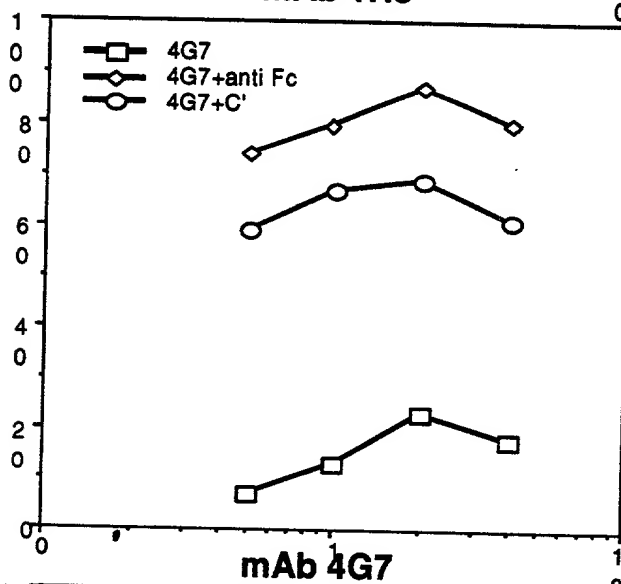
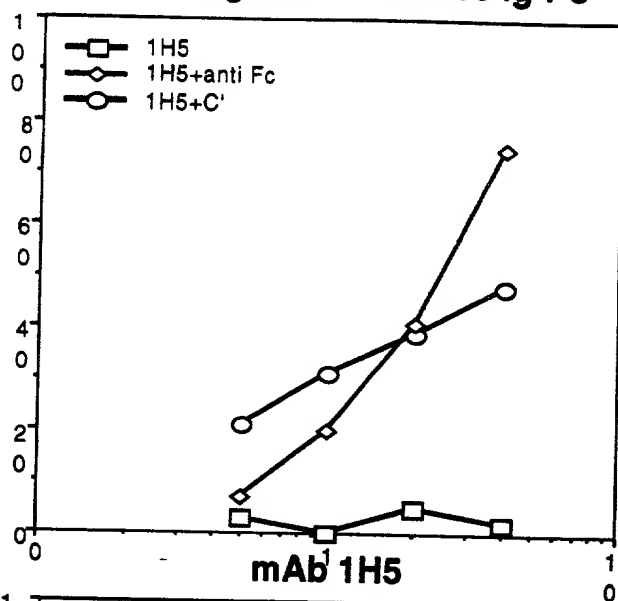


Fig. 8A

Counts

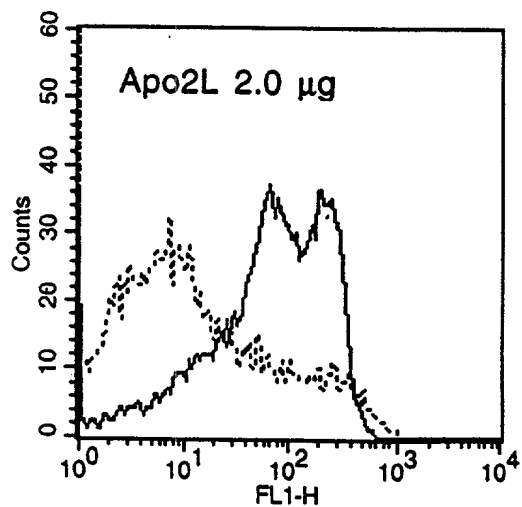
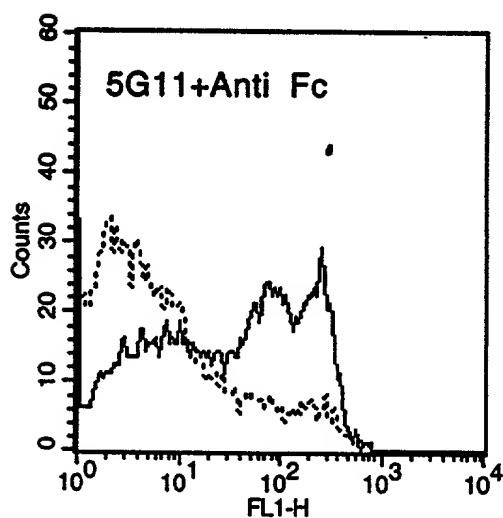
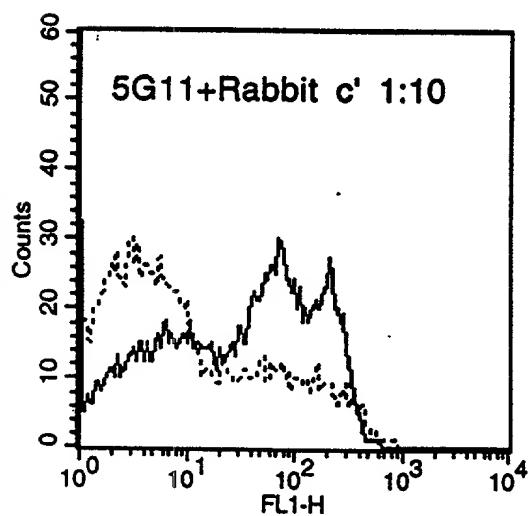
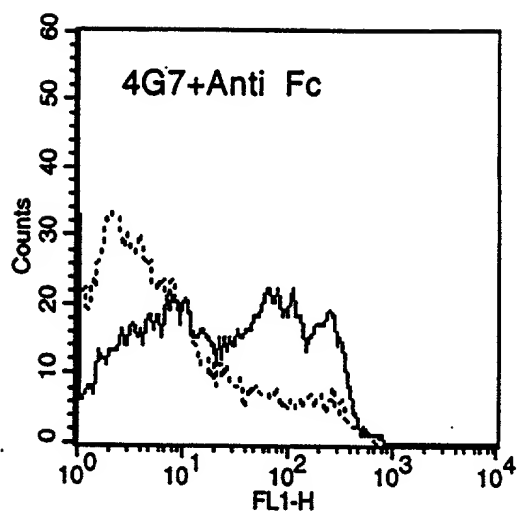
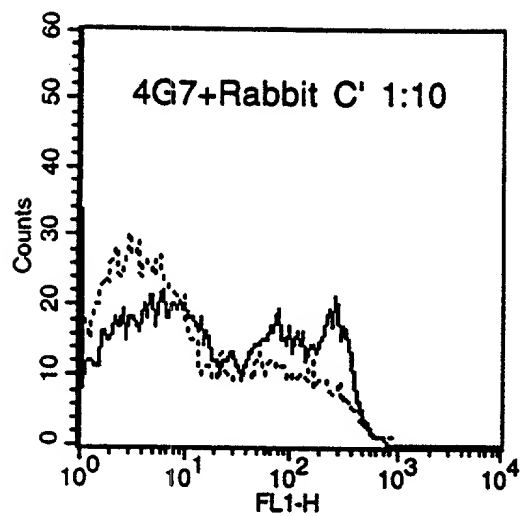


Fig. 8B

Fig. 9

plate 8-DR4 Mab

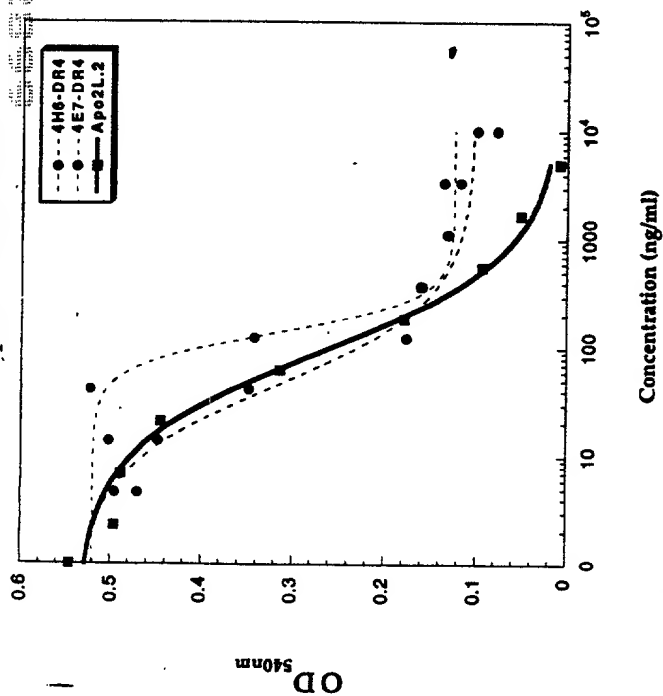


plate 9-DR4 Mab

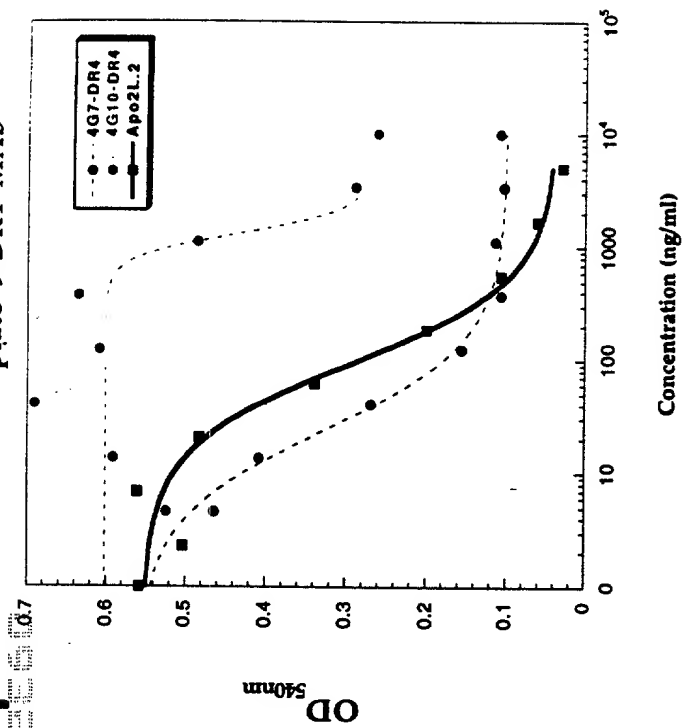


plate 7-DR4 Mab

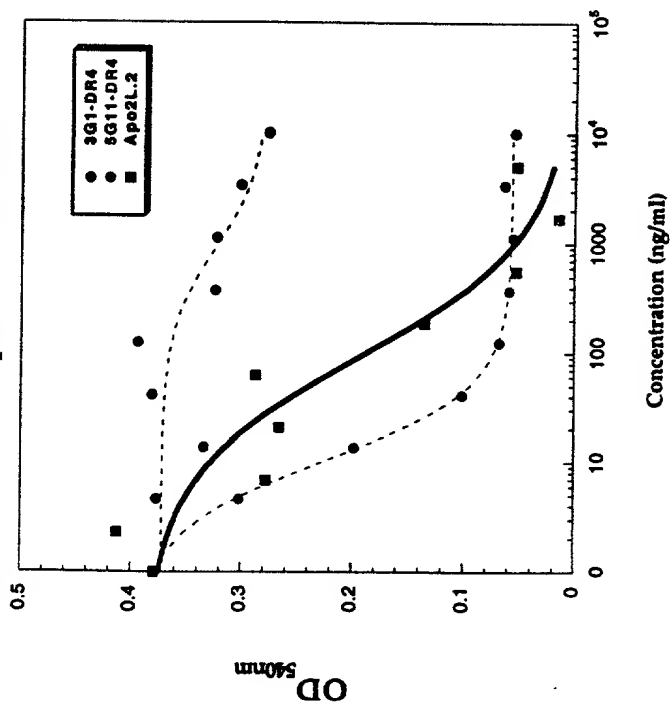
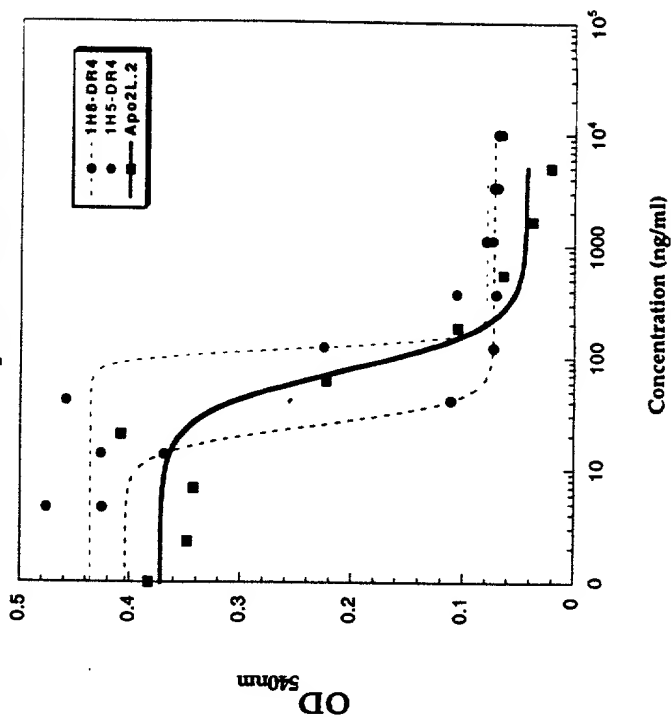


plate 6-DR4 Mab



Apoptosis of anti DR4 mAbs plus goat anti FcAb

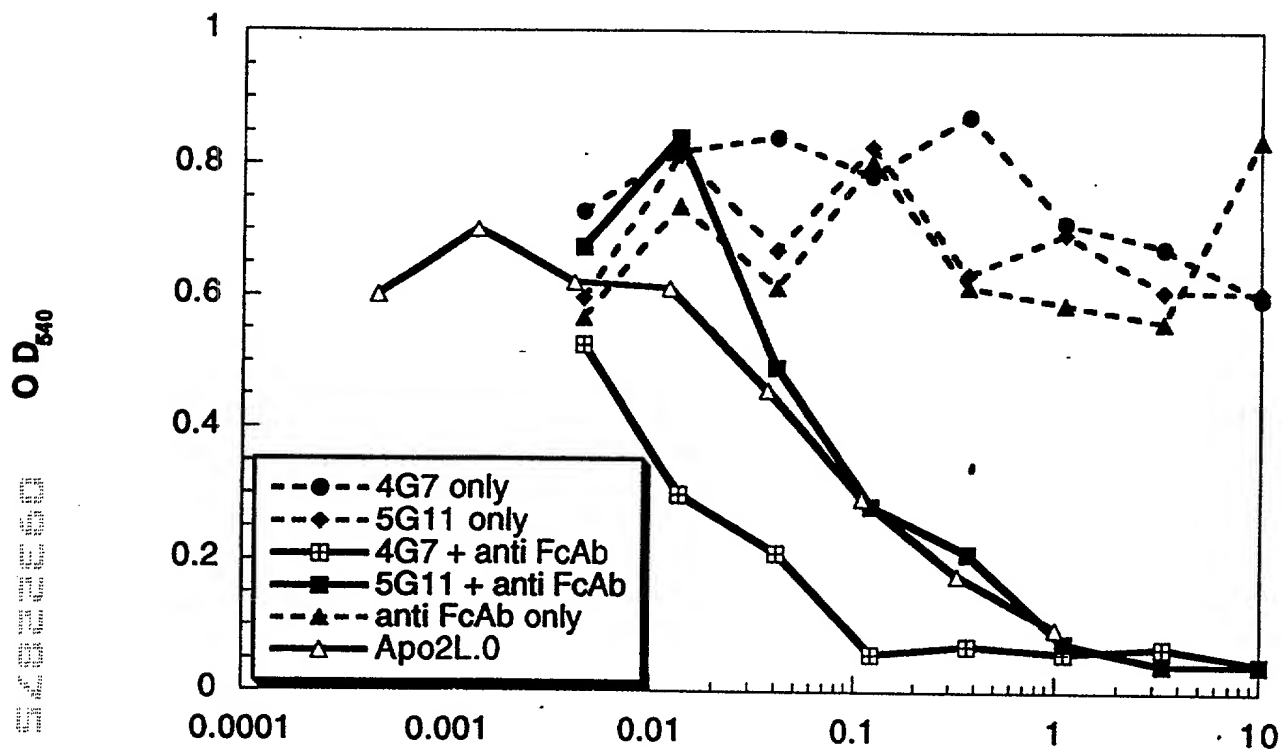


Fig.10A

Apoptosis induced by anti DR4 mAbs plus Complements

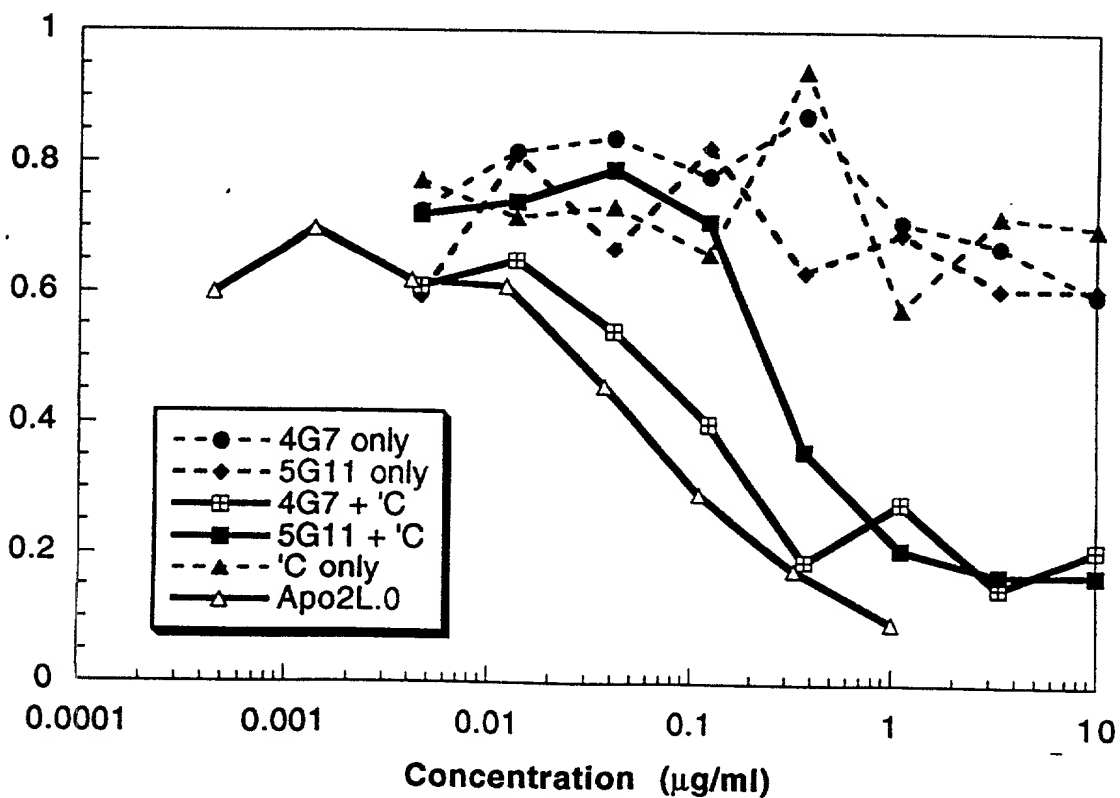
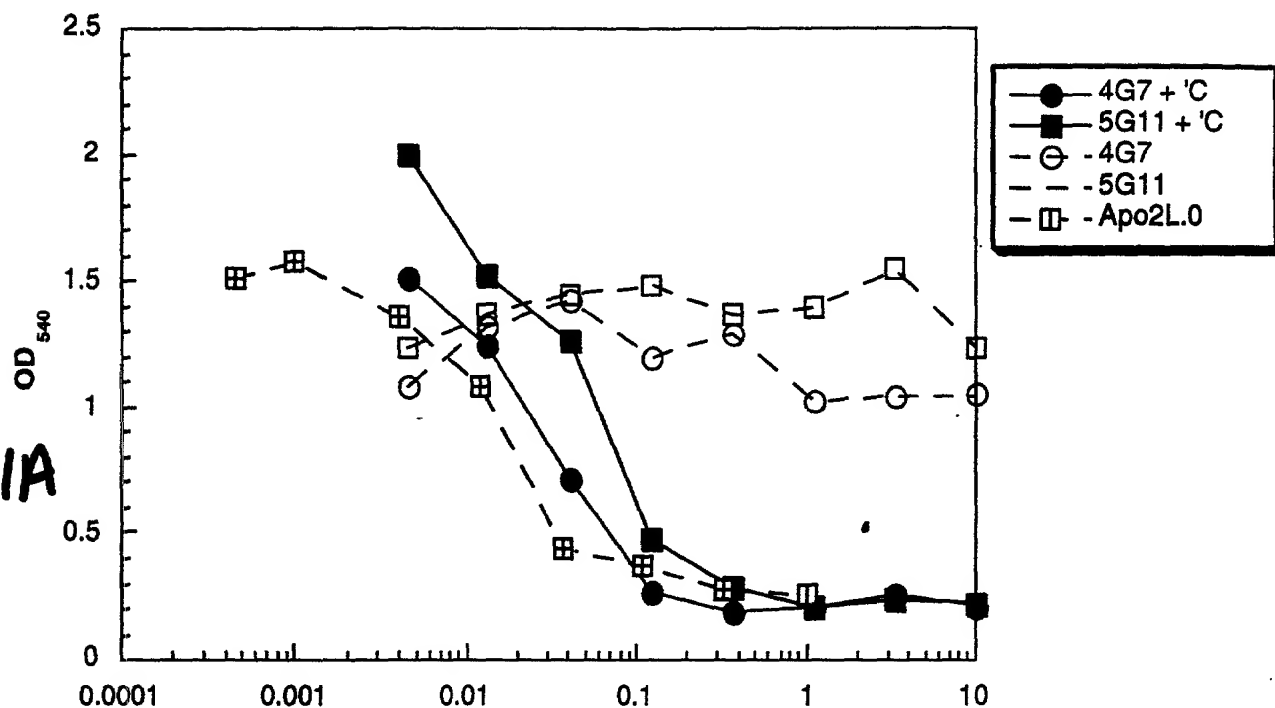
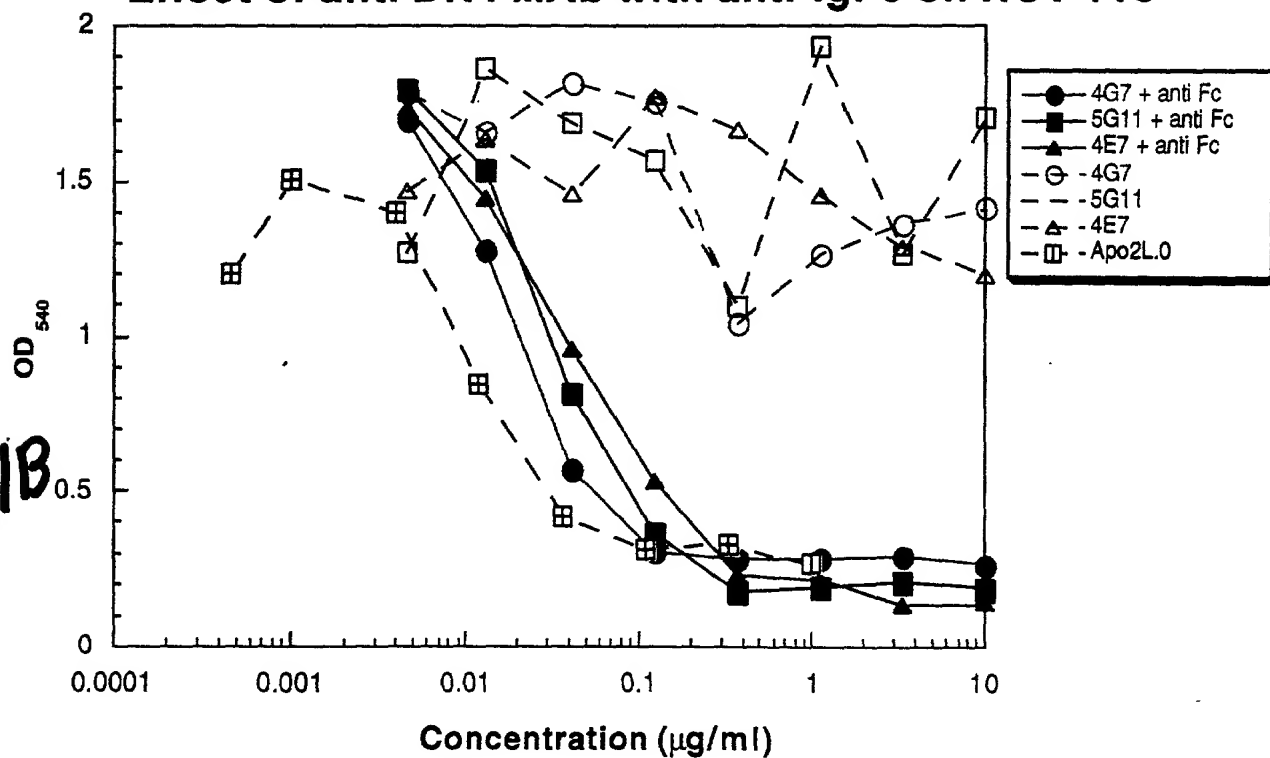


Fig.10B

Effect of anti DR4 MAb with complement on HCT 116



Effect of anti DR4 MAb with anti-IgFc on HCT 116



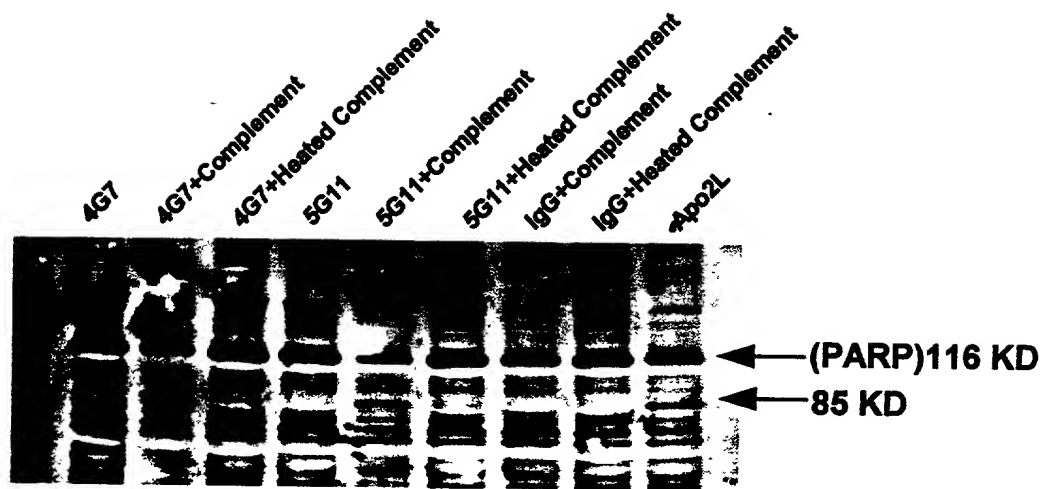


Fig. 12

Effect of anti-DR4 mAbs
on the growth of HCT-116 colon tumor

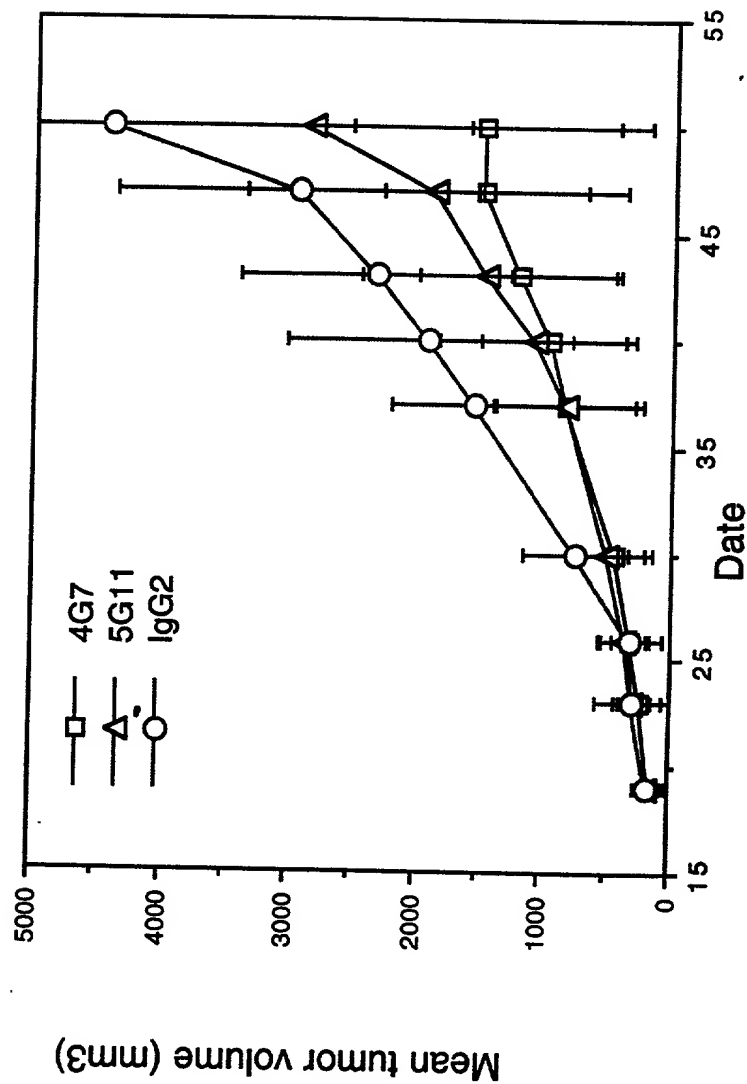


Fig. 13

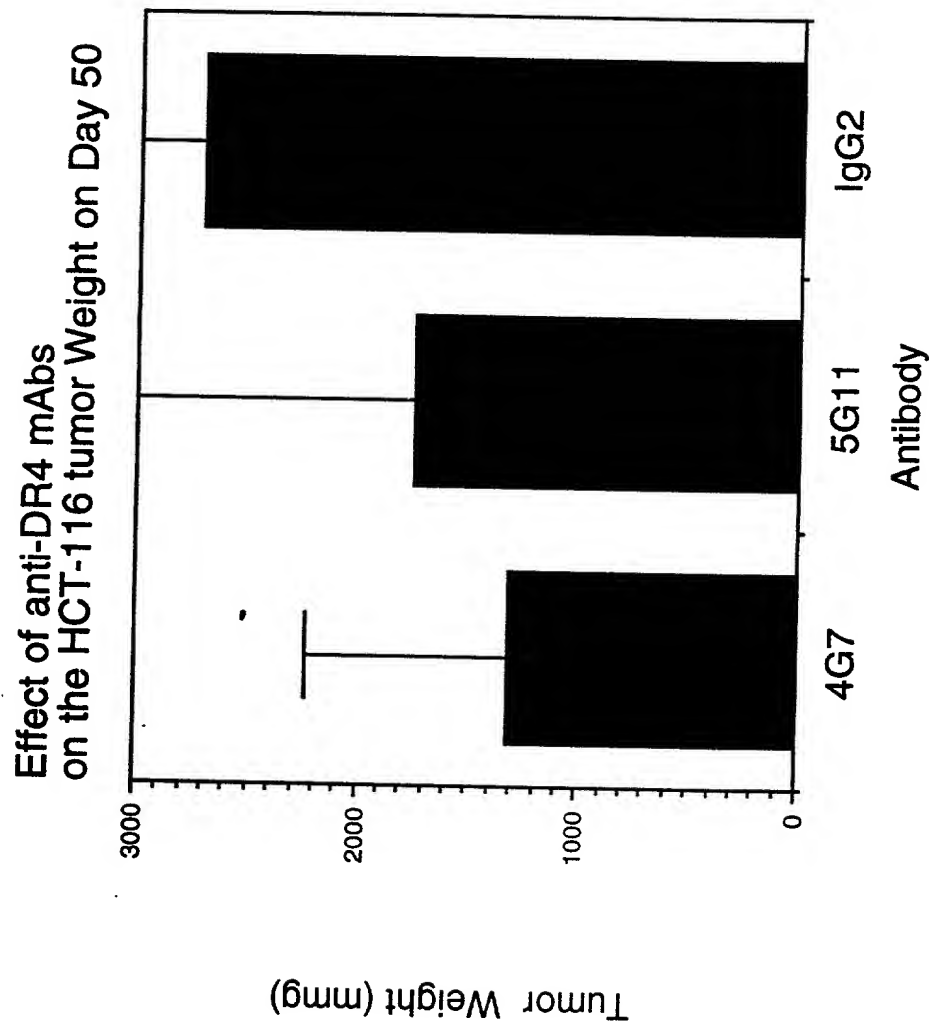
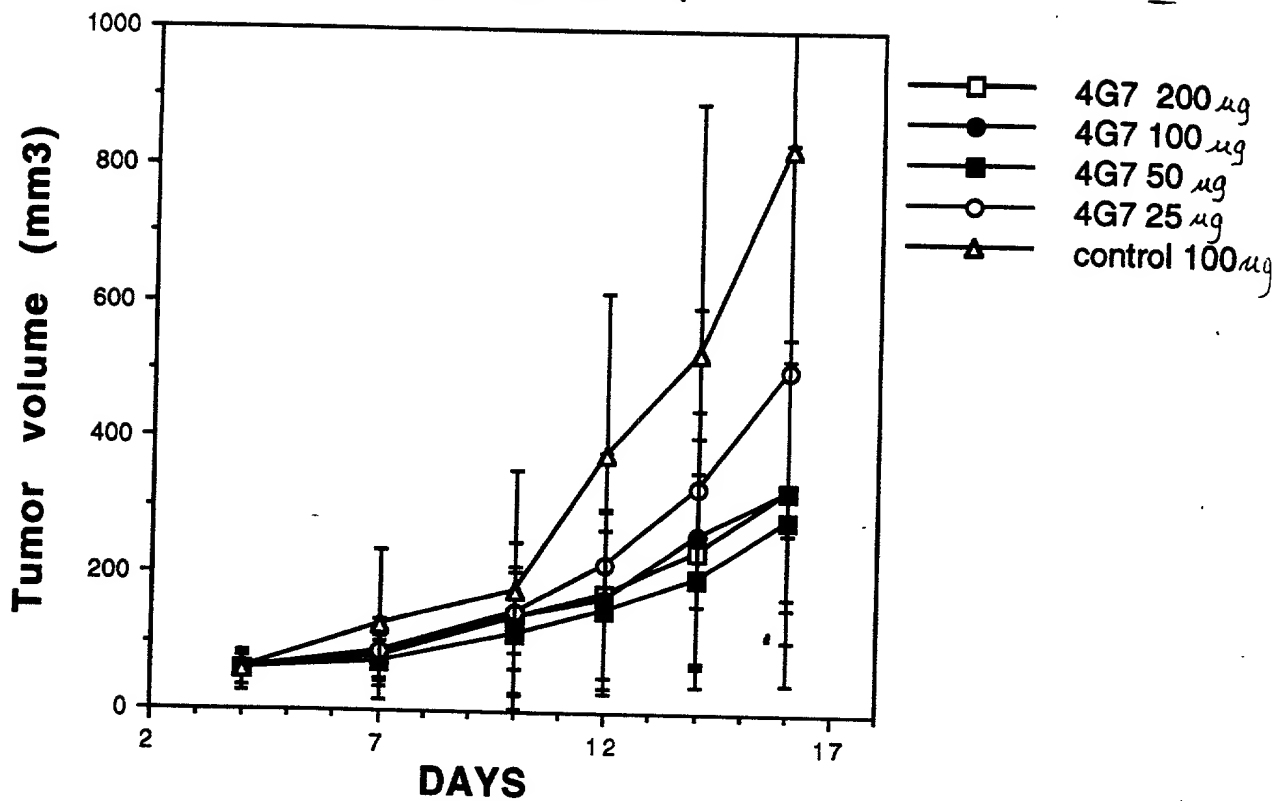


Fig. 14

Colo 205 (98-296 D)



Colo 205 (98-296C)

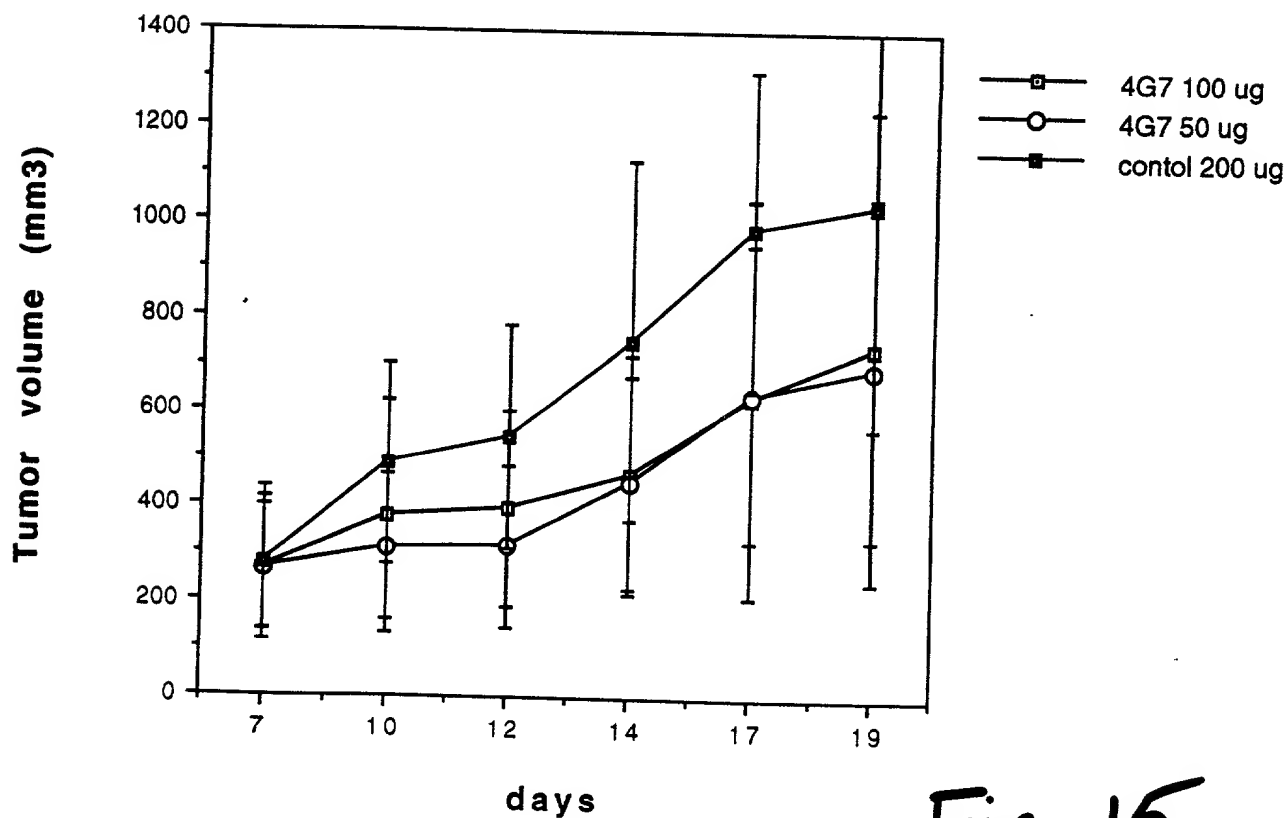


Fig. 15

Colo 205 (98-296 D)

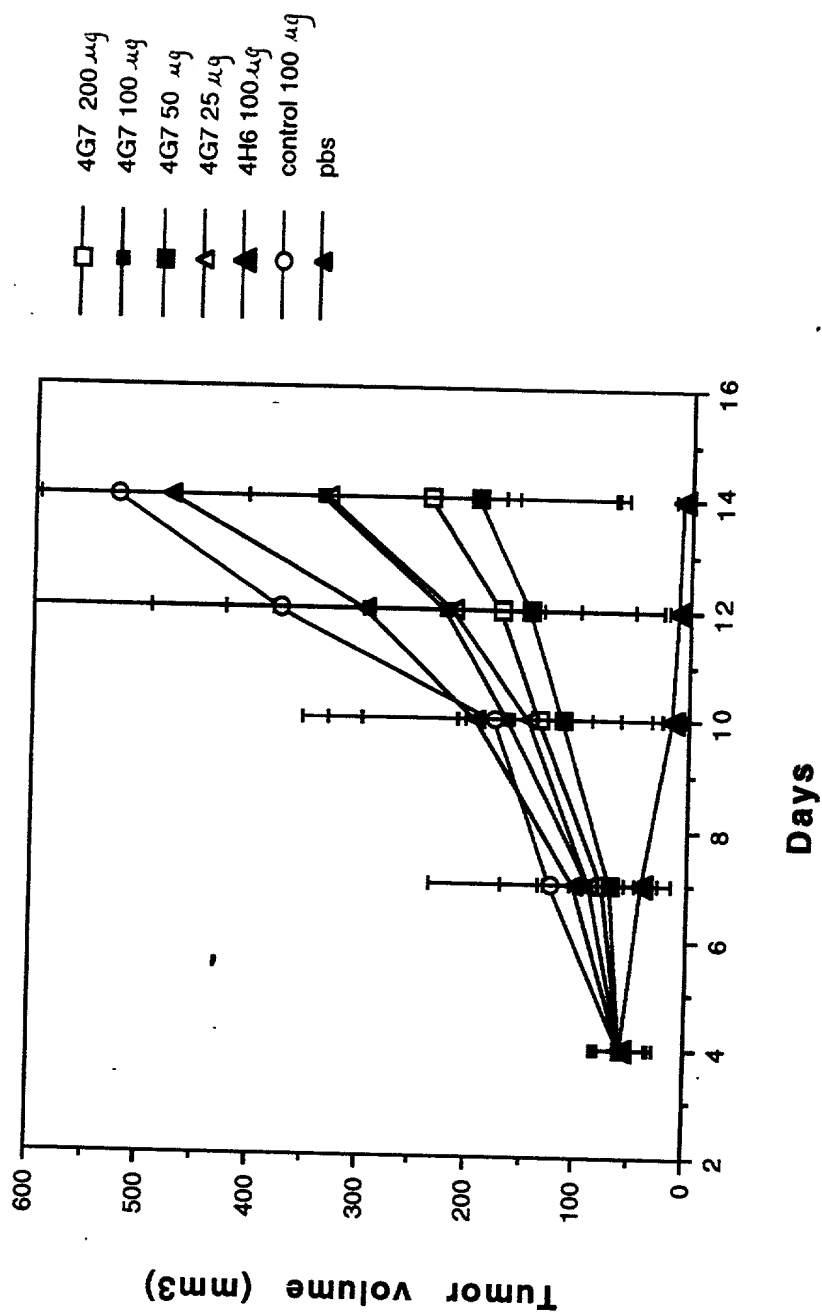


Fig. 16

General Characteristics of Anti-DR4 mAbs

Isotype	Kd-1 (pM)	Apop w/o L	Apop + &FC	Apop + C'	Block	Cross reactivity			
						DR4	DR5	DCR1	DCR2
1H5.24.9	IgG2a	-	-	-	ND	+++	-	-	-
1H8.17.5	IgG1	+	+	ND	ND	+++	-	-	-
3G1.17.2	IgG1	-	-	ND	-	+++	-	-	-
4E7.24.3	IgG1	+	+	-	-	+++	+	-	+/-
4G7.18.8	IgG2a	+	+	+	-	+++	-	-	-
4H6.17.8	IgG1	+	+	-	+	+++	+	-	-
4G10.20.6	IgG1	+	+	ND	-	+++	+	-	-
5G11.17.1	IgG2b	+	+	+	ND	+++	++	-	-

All these mAbs recognize DR4 on 9D cells and immune precipitate DR4-IgG.

w/o L : The apoptotic ability of mAbs by themselves was detected on 9D cells, skmes cells , HCT116 and colo 205

+ &FC: The apoptotic ability of mAbs was determined in combination with goat anti-mouse IgG FC.

+ C' : The apoptotic ability of mAbs was determined in the presence of rabbit complement

Degrees of binding (+) to DR5 by Mabs 4E7 and 4H6 at 10 ug/ml are 15% of the binding to DR4.

Fig . 17

MERCHANT & GOULD P.C.

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: DR4 ANTIBODIES AND USES THEREOF

The specification of which

- a. ☐ is attached hereto
b. ☒ is entitled DR4 ANTIBODIES AND USES THEREOF, having an attorney docket number of 11669.19US03.
c. ☐ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. filed and as amended on (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

- a. ☒ no such applications have been filed.
b. ☐ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)
09/237,299	25-JANUARY-1999	PENDING

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)
60/072,481	26-JANUARY-1998

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Albrecht, John W.	Reg. No. 40,481	Lacy, Paul E.	Reg. No. 38,946
Anderson, Gregg I.	Reg. No. 28,828	Larson, James A.	Reg. No. 40,443
Ansems, Gregory M.	Reg. No. 42,264	Lasky, Michael B.	Reg. No. 29,555
Batzli, Brian H.	Reg. No. 32,960	Liepa, Mara E.	Reg. No. 40,066
Beard, John L.	Reg. No. 27,612	Lindquist, Timothy A.	Reg. No. 40,701
Black, Bruce E.	Reg. No. 41,622	Lynch, David W.	Reg. No. 36,204
Blasdel, Thomas L.	Reg. No. 31,329	Marschang, Diane L.	Reg. No. 35,600
Bogucki, Raymond A.	Reg. No. 17,426	McDaniel, Karen D.	Reg. No. 37,674
Bruess, Steven C.	Reg. No. 34,130	McDonald, Daniel W.	Reg. No. 32,044
Byrne, Linda M.	Reg. No. 32,404	McIntyre, Iain A.	Reg. No. 40,337
Carlson, Alan G.	Reg. No. 25,959	Mueller, Douglas P.	Reg. No. 30,300
Caspers, Philip P.	Reg. No. 33,227	Nasiedlak, Tyler L.	Reg. No. 40,099
Chiapetta, James R.	Reg. No. 39,634	Nelson, Albin J.	Reg. No. 28,650
Clifford, John A.	Reg. No. 30,247	Pauly, Daniel M.	Reg. No. 40,123
Cochran, William W.	Reg. No. 26,652	Phillips, John B.	Reg. No. 37,206
Daignault, Ronald A.	Reg. No. 25,968	Plunkett, Theodore	Reg. No. 37,209
Daley, Dennis R.	Reg. No. 34,994	Pytel, Melissa J.	Reg. No. 41,512
Dalglish, Leslie E.	Reg. No. 40,579	Reich, John C.	Reg. No. 37,703
Daulton, Julie R.	Reg. No. 36,414	Reiland, Earl D.	Reg. No. 25,767
DeVries Smith, Katherine M.	Reg. No. 42,157	Rittmaster, Ted R.	Reg. No. 32,933
DiPietro, Mark J.	Reg. No. 28,707	Schmaltz, David G.	Reg. No. 39,828
Edell, Robert T.	Reg. No. 20,187	Schuman, Mark D.	Reg. No. 31,197
Epp Ryan, Sandra	Reg. No. 39,667	Schumann, Michael D.	Reg. No. 30,422
Funk, Steven R.	Reg. No. 37,830	Scull, Timothy B.	Reg. No. 42,137
Glance, Robert J.	Reg. No. 40,620	Sebald, Gregory A.	Reg. No. 33,280
Goffa, Charles E.	Reg. No. 26,896	Skoog, Mark T.	Reg. No. 40,178
Gorman, Alan G.	Reg. No. 38,472	Soderberg, Richard	Reg. No. P-43,352
Gould, John D.	Reg. No. 18,223	Sumner, John P.	Reg. No. 29,114
Gregson, Richard	Reg. No. 41,804	Sumners, John S.	Reg. No. 24,216
Gresens, John J.	Reg. No. 33,112	Tellekson, David K.	Reg. No. 32,314
Hamre, Curtis B.	Reg. No. 29,165	Trembath, Jon R.	Reg. No. 38,344
Hillson, Randall A.	Reg. No. 31,838	Underhill, Albert L.	Reg. No. 27,403
Holzer, Jr., Richard J.	Reg. No. 42,668	Vandenburgh, J. Derek	Reg. No. 32,179
Johnston, Scott W.	Reg. No. 39,721	Vradenburgh, Anna M.	Reg. No. 39,868
Kadiévitch, Natalie D.	Reg. No. 34,196	Welter, Paul A.	Reg. No. 20,890
Kastelic, Joseph M.	Reg. No. 37,160	Whipps, Brian	Reg. No. 43,261
Kettelberger, Denise	Reg. No. 33,924	Wickhem, J. Scot	Reg. No. 41,376
Knearl, Homer L.	Reg. No. 21,197	Williams, Douglas J.	Reg. No. 27,054
Komanduri, Janaki	Reg. No. 40,684	Witt, Jonelle	Reg. No. 41,980
Kowalchuk, Alan W.	Reg. No. 31,535	Wood, William J.	Reg. No. 42,236
Kowalchuk, Katherine M.	Reg. No. 36,848	Xu, Min S.	Reg. No. 39,536
Kubota, Glenn M.	Reg. No. 44,197		

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant & Gould P.C. to the contrary.

Please direct all correspondence in this case to Merchant & Gould P.C. at the address indicated below:

Merchant & Gould P.C.
3100 Norwest Center
90 South Seventh Street
Minneapolis, MN 55402-4131

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2 0 1	Full Name Of Inventor	Family Name CHUNTHARAPAI	First Given Name ANAN	Second Given Name
	Residence & Citizenship	City COLMA	State or Foreign Country CALIFORNIA	Country of Citizenship USA
	Post Office Address	Post Office Address 826 ELLIS DRIVE	City COLMA	State & Zip Code/Country CALIFORNIA 94015/USA
Signature of Inventor 201:				Date:
2 0 2	Full Name Of Inventor	Family Name KIM	First Given Name KYUNG	Second Given Name JIN
	Residence & Citizenship	City LOS ALTOS	State or Foreign Country CALIFORNIA	Country of Citizenship USA
	Post Office Address	Post Office Address 622 BENVENUE AVENUE	City LOS ALTOS	State & Zip Code/Country CALIFORNIA 94024/USA
Signature of Inventor 202:				Date:

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)–(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim;

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

(1) Each inventor named in the application:

(2) Each attorney or agent who prepares or prosecutes the application; and

(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.